



Sagar BHATTA

Graduated in Bachelor of Science (Engineering) in Chemical Engineering & Polymer Science

**Separation Of Bioactive Peptides From
Whey Hydrolysate Using
Electrodialysis With Ultrafiltration
Membrane: A Pilot-Scale Study And
Investigation On Process Parameters**

Dissertation for obtaining the Master degree in Membrane Engineering

Erasmus Mundus Master in Membrane Engineering

Advisor: Laurent Bazinet, Professor, Université Laval

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Jury

Chair: Isabel Coelho, Assistant Professor, UNL

Examiner(s): Partice Bacchin, Professor, UPS

Damien Quemener, Associate Professor, UM

Vlastimil Fila, Assistant Professor, UCTP

Svetlozar Velizarov, Researcher, UNL



FACULDADE DE
CIÊNCIAS E TECNOLOGIA
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Sagar BHATTA

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Abstract

A tryptic hydrolysis of whey protein isolate has generated 19 peptides from β -lactoglobulin source, out of 27 peptide sequences detected under HPLC-MS. Amongst 19 peptides, 12 were detected as anionic peptides and 5 as cationic peptides. The aim of this work was to investigate the process parameters for fractionating bioactive peptides from protein hydrolysate by pilot-scale electrodialysis with ultrafiltration membrane (EDUF) unit. Preliminary tests were performed to evaluate process parameters. A pressure-flow relation was studied for establishing no transmembrane pressure. Protein hydrolysate was fractionated during 60 minutes by EDUF on a pilot-scale EUR6 module. Under a constant pH of 6 and electric field strength of 0.7 V/cm, peptide migration rates of $0.57 \pm 0.25 \text{ g/m}^2\text{h}$ and $0.29 \pm 0.09 \text{ g/m}^2\text{h}$ were achieved in anionic and cationic peptides recovery compartments respectively. An experiment was also further investigated under two electric field conditions: pulsed electric field (PEF) and reverse polarity (RP) to observe the effect on migration rate and selectivity. Total migration rates were found to be 0.51 ± 0.05 and $0.38 \pm 0.15 \text{ g/m}^2\text{h}$ under PEF and RP conditions, respectively. An application of PEF and RP were able to separate selectively few of peptides. Peptide migration rate and selective separation of peptides found to be strongly depended on electric field strength and pressure/flow rate in each compartment. It is the low electric field and relatively higher pressure that hinders the simultaneous separation of anionic and cationic peptides in their respective compartment. To our knowledge, it was the first attempt to study separation of bioactive peptides from whey protein isolate in a pilot scale EDUF module.

Keywords: Whey protein hydrolysate, β -lactoglobulin, Bioactive peptides, Electrodialysis, Ultrafiltration membrane, Peptide fractionation.

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Abbreviations

EDUF: Electrodialysis with ultrafiltration membrane

ED: Electrodialysis

UFM: Ultrafiltration membrane

NF: Nanofiltration

MWCO: Molecular weight cut-off

PES: Polyether sulfone

IEM: Ion-exchange membrane

AEM: Anion-exchange membrane

CEM: Cation-exchange membrane

A_{RC}^- : Anionic peptides recovery compartment

C_{RC}^+ : Cationic peptides recovery compartment

pI: Isoelectric point

β -Lg: Beta-Lactoglobulin

BSA: Bovine serum albumin

ACE: Angiotensin converting enzyme

TMP: Transmembrane pressure

HPLC: High performance liquid chromatography

MS: Mass spectroscopy

PEF: Pulsed electric field

RP: Reverse polarity

STMR: Stirred-tank membrane reactor

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CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Background and motivation

The use of specific food for the prevention or treatment of diseases in human societies dated back to the ancient ages. Nearly 2500 years ago, Hippocrates stated, “let food be thy medicine and medicine be thy food.” In an ancient time, Indian sage Charaka worked with medicinal qualities of foods and herbs for healing the malfunctioning of the body, which is nowadays called Ayurveda [1]. In the modern society, there is increasing number of health problems like obesity, cancer and cardiovascular diseases which are related to the unhealthy dietary behaviors. Thus, there has been a widespread upsurge of interest among scientists in functional foods and nutraceuticals. Consumers have been increasingly oriented towards healthy foods with a consciousness of interrelation between diet and health. A food that contains bioactive ingredient such as oligosaccharides, minerals, polyunsaturated fatty acids and fibers are considered as functional foods. Therefore, bioactive compounds that are largely found in natural food and plant have been extensively studied.

In the last decade, research has increased in the field of bioactive peptides from milk proteins. Such peptides cover a range of biological and physiological properties that may be important in development of new product of added value. This value added product could be used in replacement of synthetic drugs, particularly in the treatment of chronic diseases such as hypertension, cancer and obesity. Several reviews on bioactive peptides derived from the milk protein have been published recently. For instances, bioactive peptides produced from dairy [2] [3]; ACE inhibitory and opioid peptides from whey [4]. These peptides are encoded within the sequence of native protein. Various methods for isolation and production of bioactive peptides from their natural sources are attempted without losing its bioactivity. It may be generated *in vitro* by chemical and enzymatic hydrolysis. While enzymatic hydrolysis is more preferable because of high reaction specificity and reduced waste generation. Amongst other enzymes, hydrolysis with trypsin is the mostly used for generating peptides from whey protein since it produces wider range of bioactive peptides [3]. But these generated peptides are low in concentration and hence, their purification and concentration by various separation techniques are needed.

Membrane processes showed a prominent role in the separation of bioactive peptides from hydrolysate. Several studies were reported on the use of enzymatic membrane reactors for the continuous production of protein hydrolysates and peptides separation [5],[6]. In addition, pressure driven processes like ultrafiltration and nanofiltration have also been used to study the separation of peptides based on their molecular sizes. A further enrichment of peptides has been reported by using stepped-UF method [7], where two different MWCO of membrane were used. However, most of the pressure-driven processes have significant limitations such as fouling and poor selectivity. It also lacks the simultaneous separation of cationic and anionic peptides since the pressure-driven separation is mostly governed by size difference than charge of peptides.

Lapointe et al. (2005) reported the selective separation of cationic peptides from β -lactoglobulin using electrical field in nanofiltration membrane. Later, a simultaneous fractionation of cationic and anionic peptides has been successfully achieved by Poulin et al.[8] using electrodialysis with ultrafiltration membrane (EDUF), a process patented by Dr.

Bazinet [9]. Laboratory scale-EDUFs have been successfully investigated for separation and isolation of various bioactive peptides like anticancer and antibacterial from snow crab byproducts, antidiabetic and antihypertensive peptides from flaxseed and antioxidant polyphenol from cranberry juice. Moreover, EDUF has an advantage of lower fouling compared to conventional pressure driven process and no change in selectivity due to cake layer formation.

Despite of its wide application and perspective in the fractionation of bioactive peptides from various food sources, an industrial application of EDUF was still unexplored. In order to use EDUF successfully for an industrial application, an extensive study of process parameter on pilot scale is necessary. A scaling up of laboratory scale can be achieved by studying the influence of electric field strength, MWCO of ultrafiltration membrane (UFM), nature and conductivity of UFM and configuration in pilot-scale. Different approaches of electric field conditions like reverse polarity (RP) and pulsed electric field (PEF) could be used for minimizing the fouling and scaling in the membrane and study the effect on membrane selectivity. A next step should account for the economic analysis of the process to be used commercially in a large-scale for the separation of bioactive compounds. This present work is to study a separation of peptides generated from tryptic hydrolysis of β -lactoglobulin using pilot-scale EDUF and observe the effect of various electric field on the performance of EDUF process.

1.2 Research objectives

As explained above, the aim of the present work is to produce peptides from an enzymatic hydrolysis of β -lactoglobulin and to investigate the feasibility of pilot-scale EDUF for the simultaneous separation of bioactive peptides using ultrafiltration membranes stacked in a batch recirculation process. The objectives of the current work were to:

- (i) perform a preliminary test on pilot-scale EDUF for establishing pressure-flow relation in each compartment,
- (ii) study the effect of electrodialytic parameters for the isolation of bioactive peptides from enzymatic hydrolysate of whey protein isolate and,
- (iii) investigate the effect of electric fields on the performance of EDUF process,
- (iv) characterize the peptides in feed hydrolysate and recovery compartments after EDUF process.

CHAPTER 2

LITERATURE REVIEW

2. Literature Review

2.1 Functional food and Nutraceuticals

2.1.1 Definition

The concept of functional foods has been widely spread as consumers are increasingly aware of the important link between diet and health. A balanced diet plays a vital role in combating chronic diseases and enhancing health. Thus an unbalanced diet and unhealthy eating habits have become major challenges in most of the countries around the world. It has caused a rise in conditions such as obesity, cancer, diabetes, cardiovascular diseases (CVD), osteoarthritis and allergies. For instance, 11 million people in the USA have type-2 diabetes [10]. Moreover, Soedamah-Muthu et al.[11] reported that cardiovascular diseases are the primary causes of death in the Western countries.

Research in the field of functional foods and nutraceuticals has been extensively carried out in the recent years. According to the Institute of Medicine's Food and Nutrition Board, functional food is defined as "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains". On the other hand nutraceuticals, as defined by Wildman (2000), "are naturally occurring substances in foods which have been found to be effective in the prevention of treatment of one or more diseases, or the improvement of physiological performance, thereby enhancing human health [12]." However there is yet no universal definition of functional food and nutraceuticals.

As understood commonly, functional foods and nutraceuticals include the whole spectrum of foods with disease-preventive and health-promoting properties. Functional foods may be enriched with a specific health beneficial component, vitamin and mineral supplements, herbs, phytochemicals, and probiotics. Nutraceuticals can be derived from plant, animal, and microbial sources, including those from aquatic environment. Among various nutrients found from animal and plant sources, proteins have been broadly identified as sources of physiologically active peptides.

2.1.2 Protein-precursor of bioactive peptides

Bioactive peptides are those protein fragments that have a positive impact on body functions or conditions that may ultimately influence health. The bio-functionality of these peptides depends on their inherent amino acid composition and sequence. The size of peptides usually varies from 2 to 20 amino acids. These peptides are mostly inactive within the sequence of parent protein and can be released during gastrointestinal digestion or food processing. These bioactive peptides carry a wide range of biological, functional and nutritional properties. Depending on the amino acid sequence, these peptides may evoke numerous biological functions in vivo affecting, for instance, the cardiovascular, endocrine, digestive, immune and nervous systems. The beneficial health effects may further be classified as antimicrobial, antioxidant, antithrombotic, antihypertensive, antimicrobial or immunomodulatory, etc [13][14].

2.1.3 Bioactive peptides from milk

Milk is a rich source of protein and also considered as an important source of bioactive peptides. Milk contains critical nutritive elements which are vital to both neonates and adults. Bioactive peptides can be produced from milk proteins through fermentation of milk, whey and ripened cheese. A considerable evidence of multifunctional capacities and bio-specific role of bioactive peptides from milk can be found in the literature ([15], [16], [17], [18]). An overview of primary classes of bioactive milk peptides, based on their specific physiological function and characteristics are presented in Table 2.1. Bioactive peptides evoke immune-defense, neurological, physiological and vasoregulatory responses (Table 2.1).

Table 2.1 Specific physiological function and characteristics of some bioactive milk peptides in milk. (adapted from Claire et al.[19])

No.	Peptide sequence	Name	AA segment	Physiological classification	Release Protease	Reference
1	FFVAP	α 1-Casokinin-5	α 1-CN (f 23–27)	ACE inhibitor	Proline endopeptidase	[20]
2	AVPYPQR	β -Casokinin-7	β -CN (f 177–183)	ACE inhibitor	Trypsin	[20]
3	YGLF	α -Lactorphin	α -LA (f 50–53)	ACE inhibitor and opioid agonist	Synthetic peptide	[21]
4	ALPMHIR	β -Lactorphin	β -LG (f 142–148)	ACE inhibitor	Trypsin	[22]
5	KVLPVPQ	Antihypertensive peptide	β -CN (f 169–174)	Antihypertensive peptide	Lactobacillus CP790 protease	[23]
6	MAIPPKKNQDK	Casoplatelin	κ -CN (f 106–116)	Antithrombotic	Trypsin & synthetic peptide	[24]
7	KDQDK	Thrombin inhibitory peptide	κ -CN glyco-macropptide (f 112-116)	Antithrombotic	Trypsin	[25]
8	KRDS	Thrombin inhibitory peptide	Lactotransferrin (f 39-42)	Antithrombotic	Pepsin	[26]
9	QMEAESISS EEIVPNSVEQK	Caseinophospho-peptide	α 1-CN (f 59-79)	Calcium binding and transport	Trypsin	[17]
10	LLY	Immunoepitope	β -CN (f 191–193)	Immunostimulatory (+)	Synthetic	[27]
11	FKCRRWQWRMK KLGAPSITCVRRA F	Lactoferricin B	Lactoferrin (f 17–41)	Immunomodulatory (+) and antimicrobial	Pepsin	[28], [29]
12	YQQPVLGPVR	β -Casokinin-10	β -CN (f 193–202)	Immunomodulatory (+/-) & ACE inhibitor	Synthetic	[30]
13	RYLGYLE	α -Casein exorphin	α 1-CN (f 90–96)	Opioid agonist	Pepsin	[31]
14	YGFQNA	Serorphin	BSA (f 399–404)	Opioid agonist	Pepsin	[32]
15	YLLF-NH ₂	β -Lactorphin	β -LG (f 102–105)	Opioid agonist and ACE inhibitor	Synthetic or Trypsin	[21]
16	YIPIQYVLSR	Casoxin C	κ -CN (f 25–34)	Opioid antagonist	Trypsin	[33]
17	YVPF PPF	Casoxin D	α 1-CN (f 158–164)	Opioid antagonist	Pepsin-chymotrypsin	[34]
18	YLGSGY-OCH ₃	Lactoferroxin A	Lactoferrin (f 318-323)	Opioid antagonist	Pepsin	[35]

In general, caseins and whey proteins are the two main groups of protein in bovine milk. Each of the sub-fractions found in casein or whey has its own unique biological properties. Caseins are sub-divided into α -, β - and κ -caseins that comprises 80 percent of the total protein content in bovine milk. Whey comprises a heterogeneous group of proteins that remains in the supernatant after the precipitation of casein. Whey proteins are composed of β -lactoglobulin, α -lactalbumin, immunoglobulins (IgGs), glycomacropeptides, bovine serum albumin (BSA), and minor proteins such as lactoperoxidase, lysozyme and lactoferrin. These proteins have a compact globular structure. These proteins are dephosphorylated, insensitive to Ca^{2+} and easily denatured upon heating at temperature 90°C [36]. Typical physico-chemical properties of whey protein are tabulated in Table 2.2.

Table 2.2 Typical physico-chemical properties of whey protein (Adapted from Zydney et al,2000)

Proteins	Concentration (g/L)	MW (kDa)	Isoelectric point (pI)
$\beta - Lg$	3-4	18.4	5.2
$\alpha - La$	1.5	14.2	4.7-5.1
BSA	0.3-0.6	69	4.7-4.9
IgG, IgA, IgM	0.6-0.9	150-1000	5.5-8.3
Lactoperoxidase	0.006	89	9.6
Lactoferrin	0.05	78	8.0
Protease-peptone	0.5	4-20	
Caseinomacropptide		7	

$\beta - Lg$ is one of the major source of whey proteins used for the generation of bioactive peptides by microbial fermentation or enzymatic hydrolysis [3], [38]. Bioactivities of these peptides are encoded within the sequence of native protein precursors that can be generated in vitro by enzymatic hydrolysis which further can be purified by various separation techniques and assayed for bioactivity. Over the last decades, various proteases were used to produce bioactive peptides sequences from whey protein. The most commonly used protease for enzymatic digestion of $\beta - Lg$ is trypsin. A tryptic digestion of $\beta - Lg$ can produce peptides sequences (f15-20, f102-105 and f142-148) which possess ACE inhibitory activity [22]. In addition to ACE inhibitory activity, trypsin can release hypocholesterolemic peptides [39] of sequence f71-75, bactericidal peptides [40] of sequence f15-20 and f92-100, and peptide sequence f102-105 having opioid activity [17]. These bioactive molecules are usually low in concentration and present in complex matrices, therefore it is necessary to use a suitable method for production and fractionation of these bioactive peptides of interest.

2.2 Production and fractionation of bioactive peptides

There is increasing commercial interest in the production of bioactive peptides from various sources. There is a need to develop technologies which retain or even enhance the activity of bioactive peptides in food systems.

2.2.1 Production of bioactive peptides

Biologically active peptides can mainly be produced from milk proteins by microbial fermentation of milk, direct synthesis & DNA recombinant technology and enzymatic hydrolysis. Amongst the various methods, enzymatic hydrolysis is the most commonly and widely used method for production of bioactive peptides from milk. Sometimes, a combination of these methods can be used to produce short bioactive peptides.

2.2.1.1 Enzymatic hydrolysis

Enzymatic hydrolysis is so far the most important and preferred method for the production of bioactive peptides. It involves proteolytic enzymes (also called proteases) for the digestion of native proteins to produce shorter peptides. There are several advantages of proteolytic hydrolysis over chemical hydrolysis [41] which are pointed below:

- i) defined product stereochemistry due to the high substrate and reaction specificity of biocatalyst,
- ii) mild reaction conditions, and
- iii) reduced waste generation.

The function of generated peptides from enzymatic hydrolysis mainly depends on the primary sequence of the protein substrate, the specificity of enzyme and digestive conditions. The digestive conditions of hydrolysis depend on pH, temperature, reaction time and degree of hydrolysis [42]. Trypsin, chymotrypsin, pepsin, alcalase, etc are some of the enzymes used to generate bioactive peptides from proteins. Amongst these enzymes, trypsin is the most widely used enzyme for digestion of protein from bovine milk. It is because of its tendency to generate more bioactive peptides even without combining with other enzymes (Weiter et al, 1996). Whey proteins mainly containing β -lactoglobulin and α -lactalbumin are digested by trypsin to produce important bioactive peptides. Trypsin generated bioactive peptides from whey have not only ACE inhibitory properties but also antibacterial activity [22] and hypocholesterolemic activity [39]. However hydrolysis of native proteins from whey leads to the formation of a complex mixture of peptides, non-hydrolyzed protein fraction and enzyme. In addition, the generated peptides after enzymatic hydrolysis are usually low in concentration. Therefore, an appropriate fractionation and purification method is important for recovery and concentration of peptides of interest for an industrial development.

2.2.2 Separation and purification of bioactive peptides

An efficient technique is crucial to separate and fractionate the bioactive peptides produced either from enzymatic hydrolysis or microbial fermentation method.

2.2.2.1 Chromatographic techniques

Novel membrane separation and ion-exchange chromatographic methods are employed by the emerging dairy ingredient industry. Chromatographic techniques (CTs) used mainly for

peptide purification are ion-exchange chromatography (IEC), size-exclusion chromatography (SEC), affinity chromatography, etc. CTs have advantages of higher sensitivity, purity and quality of final resultant product. These chromatography based techniques are found in industrial scale mostly in pharmaceuticals industry where high purity product is needed. However, it is limited to laboratory research purposes in food industry. Since they are very expensive, time consuming, applicable to only small sample volume and use a large quantity of solvents. Industrial-scale technologies suitable for the commercial production of bioactive whey peptides are consequently vital. Hence, a separation process that can be easily scaled-up for an industrial-scale at low cost using no or less solvent for the peptides separation and purification is needed. This need, therefore, can be achieved by using membrane-based processes.

2.2.2.2 Pressure-driven membrane processes

A number of membrane technologies have been developed and implemented in dairy processing since 1970s. Membrane separation processes were popular in a short span of time due to the fact that they are green technologies, use no solvent and need lower energy input in contrast to conventional methods. In a pressure driven membrane process, membrane pore size and size of particles are key factors since separation is based on size of particles applying a pressure gradient as a driving force. Pressure driven membrane separation processes include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). Among these, UF and NF are normally used for the separation of bioactive peptides from food and/or milk products.

Enzymatic membrane reactors (EMRs) have been widely studied for the continuous production of protein hydrolysates and peptides. Hydrolysis has been carried out in batch or continuous process using different combinations of membrane reactors and ultrafiltration membranes. Several studies have been reported on the production of bioactive peptides. For instances, Visser et al. [6] investigated continuous production and isolation of peptide fractions from plasmin-degraded β -casein using enzyme recycled reactor. This process has limitation of operating for short period of time as membrane fouling and loss of enzyme activity were observed after longer conversion periods. In addition, Bordenave et al. [5] used an ultrafiltration EMR to concentrate β -lactoglobulin from goat's whey and recover α -lactalbumin derived peptides. β -Lactoglobulin was concentrated in the retentate while peptides from pepsin hydrolysis were obtained in permeate. They also suggested that a smaller molecular weight cut-off (MWCO) of membrane should be used for better fractionation of the peptides.

Bouhallab et al. [43] observed the effect of UF membrane area and their nature when stirred-tank membrane reactor (STMR) was used for the extraction of an immunomodulatory peptide from a bovine β -casein using chymosin as protease. They observed that feasibility of the process mainly depends on UFM area and its nature. Membrane recycled reactor was applied by Perea et al.[44] to hydrolyze whey proteins by alcalase considering to obtain higher conversion and productivity compared to batch process. However they found that the main limitation with this process was the membrane fouling resulting in reduction of productivity. Similarly, other researches were carried out using an immobilized enzyme reactor for partial hydrolysis of whey protein concentrate on controlling the degree of hydrolysis (DH), below

10% to minimize bitterness of hydrolysates. The resulting product contained a mixture of protein and peptides with lower ACE inhibitory activity. However, the main limitations of using membrane reactors employing UFM were membrane fouling with a subsequent decrease in productivity.

In order to use peptides as nutraceuticals, further purification and fractionation of hydrolysates will be required. In the literature, the enrichment of peptides has been found to be carried out mainly by ultrafiltration methods. Turgeon et al. [7] utilized a stepped UF method to limit the range of peptides to the narrow range. They used ultrafiltration membrane with MWCO of 1 and 30 kDa to enrich ACE inhibitory peptides from α -lactoglobulin and β -lactoglobulin. Gauthier and Pouliot (2003) employed a two-step ultrafiltration process; in the first step using 30 kDa membrane and second step using 1 kDa NF membrane for non-hydrolyzed protein separation and peptides fractionation respectively. Similarly, Butylina et al. [45] also used a combination of UF and NF for the peptides fractionation from sweet whey; first step to retain completely the whey proteins from sweet whey incorporating ultrafiltration membrane of MWCO of 10 kDa, while the resulting permeate fractions were fractionated by nanofiltration with MWCO of 1 kDa.

In previous studies, it was observed that ultrafiltration, nanofiltration and sometimes their combination were extensively used for the purification and fractionation of bioactive peptides. It was also evident that UF and NF significantly lead to a problem of membrane fouling and poor selectivity when fractionating peptides of similar sizes [46]. Bioactive peptides fractionation depending on the size was possible with pressure driven process, but it lacks the ability to separate peptides according to their charges.

2.2.2.3 Electromembrane Processes

Electromembrane processes are one of the innovative fractionation methods and include electrofiltration, which consists of the application of an electrical field in order to further improve and exploit separation by charge difference. It addressed the lacking of peptides separation depending on its charge and significant fouling on UF and NF membrane observed in pressure-driven processes. Peptides fractionation by electromembrane procedure involves a concept of transport of charged peptides by using electrical field.

In 1999, Recio and Vesser [47] used laboratory-scale ion-exchange membrane for the separation of lactoferrin from whey proteins. They achieved a yield of 50%, but it took 24 hours to complete and found to be limited for scalability of this process. Lapointe et al. [48] exploited the application of electrical field in nanofiltration membrane for selective separation of cationic peptides from a tryptic hydrolysate of β -lactoglobulin. However this method lacks an electrolyte recirculation compartment that could prevent pH changes caused by electrolysis of water at the electrodes. This could lead to bioactive peptides susceptible to oxidation when comes in contact with electrodes [49].

An innovative method “electrodialysis with ultrafiltration membrane” (EDUF) for the separation of bioactive peptides has recently been emerged and patented by Bazinet et al. [9]. EDUF is a batch process which allows the migration of molecules according to their charges

and molecular weights, where migration of peptides are solely driven by applied electric field. EDUF configuration consists of ultrafiltration (UFM) and ion-exchange membranes (IEM) stacked in a conventional electrodialysis (ED) cell. Therefore, EDUF process combines the charge selectivity of conventional ED process with size exclusion characteristic of UF membranes [49]. A typical EDUF cell is generally composed of a feed solution compartment corresponding to the solution to be separated and two recovery compartments for anionic (A_{RC}^-) and cationic peptides (C_{RC}^+) which contain a salt solution (KCl) for electrical current circulation. The same or different MWCO of UFM can be placed in between ion exchange membranes [50]. There is no transmembrane pressure (TMP) applied during this process.

This technology has been found effective in numerous potential applications in the food and bio-pharmaceutical industry for the separation and the recovery of bioactive compounds. Poulin et al. (2006) studied a laboratory-scale EDUF for the simultaneous separation of bioactive peptides from β -lactoglobulin hydrolysate using a configuration of one UFM (20 kDa) between two IEM. Among 40 peptides present in the hydrolysate, 13 peptides were selectively fractionated in different compartments of EDUF cell based on their charges [8]. More recently, Doyen et al. (2011) used two UFM between IEMs where they reported an anticancer peptide fraction from snow crab byproduct hydrolysate after a selective separation by EDUF with 20 kDa MWCO ultrafiltration membrane. In another study of Doyen et al. (2012), they used two different MWCO of UF (20 kDa and 50 kDa) under electric field strength of 2 and 14 V/cm to recover and concentrate an antibacterial fraction from the snow crab byproduct hydrolysate. In the study made by Roblet et al (2013), it is found that pH modulation can bring an efficient way to concentrate the low molecular weight peptides of 400 Da in the cationic recovery compartment and to limit the diversity of peptides recovered in the anionic peptides recovery compartment. They studied under three controlled pH conditions of 3, 6 and 9.

When compared to conventional technologies, EDUF has many advantages like: (a) it is a green technology since no solvent is used, (b) high selectivity of the process since it separates bioactive peptides based on size/charge in one operation, (c) reduced membrane fouling when charged molecules are only allowed to pass through membrane under applied electric field, unlike pressure driven process, (d) scale-up is simpler by stacking membranes according to the required production and (e) it can be easily integrated with an existing industrial production line.

EDUF technique has showed potential applications for diverse raw matrices, mainly for the separation and purification of bioactive peptides/compounds. Until now EDUF has demonstrated its novelty to fractionate several bioactive compounds such as antioxidant peptides from soy-protein hydrolysate [46], chitosan oligomers [51], [52], antihypertensive peptide from alfalfa white protein hydrolysate [53], antioxidant polyphenols from cranberry juice [54]–[56], antidiabetic and antihypertensive peptides from flaxseed protein hydrolysate [57]. It proved the versatility of EDUF technology is not only limited to separation of bioactive compound from protein source but also from other food sources.

However, a deterioration of process performance due to membrane fouling is a frequently observed problem. As a result, electrodialysis system are often operated in reverse mode, where the polarity of the electrodes is reversed to change the direction of ion movement [58], [59]. Another method so-called pulsed electric field (PEF) is nowadays being used for

minimizing the effect of membrane fouling and scaling. Successful uses of PEF for reducing membrane fouling were reported by Ruiz et al.[60] for protein deposit and Cifuentes-Araya et al.[61] for scaling.

EDUF has been successfully used in the basic laboratory research for bioactive molecules separation and purification. However, scale-up of laboratory scale for an industrial application has not yet been fully examined. Pilot-scale study should be studied to investigate the process parameters before testing an EDUF system at a large scale to carry-out a complete economic analysis of the process.

CHAPTER 3

MATERIALS AND METHODS

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals

BiPro, whey protein isolate containing bovine beta-Lactoglobulin (β -Lg) with ≥ 90 % purity was purchased from Davisco (MN, USA) and bovine pancreatic trypsin, with an activity equal to 11,680 BAEE (*N* α -Benzoyl-L-Arginine-Ethyl-Ester) unit per mg proteins from Sigma-Aldrich (St. Louis, MO, USA). One BAEE unit produces ΔA_{253} of 0.001 per minutes in 3.2 mL at pH 7.6 at 25 °C. HCl, NaOH and NaCl were obtained from Fischer Scientific (Montréal, Canada). KCl was provided by VWR International and Na₂SO₄ by Anachemia Canada Co. (Montréal, Canada).

3.1.2 Membranes

Polyether Sulphone (PES) ultrafiltration membranes (UFMs) with molecular weight cut-off (MWCO) of 100 kDa were purchased from Synder Filtration (USA). Neosepta CMX-SB cationic membranes (CEM) and Neosepta AMX-SB anionic membranes (AEM) were provided by Tokuyama Soda Ltd. (Tokyo, Japan). The physicochemical properties of membranes are enlisted in Table 3.1.

Table 3.1 Physicochemical properties of membranes.

Type of membrane used	Physicochemical properties	
	<i>Thickness (mm)</i>	<i>Conductivity (mS/cm)</i>
AEM	0.14	9.20
CEM	0.17	12.06
UFM	0.177 \pm 0.002	5.88 \pm 0.200

3.2 EDUF cell configuration

The electrodialysis cell used in this experiment was an EUR6 model, manufactured and configured by Eurodia with a total effective surface area of 0.734 m². Electrodialysis with ultrafiltration (EDUF) cell consisted of 13 stacks each containing two UFMs sandwiched between ion-exchange membranes (IEMs) as shown in Figure 3.1. Both electrodes (anode and cathode) were made of nickel. A closed flow circulation was maintained with four external compartments using four centrifugal pumps with four external reservoirs. The cell was configured for a simultaneous separation of anionic and cationic peptides. A 3L of feed solution (hydrolysate of BiPro, 10 g/L) in feed compartment was circulated between two UFMs with filtration side facing towards the feed compartment. Next two compartments,

each containing 3L of KCl solution (2 g/L) were dedicated to the recovery of anionic A_{RC}^- peptides and cationic C_{RC}^+ peptides. The last compartment (electrolyte compartment) for the electrode rinsing solution contained 3L of Na_2SO_4 (20 g/L).

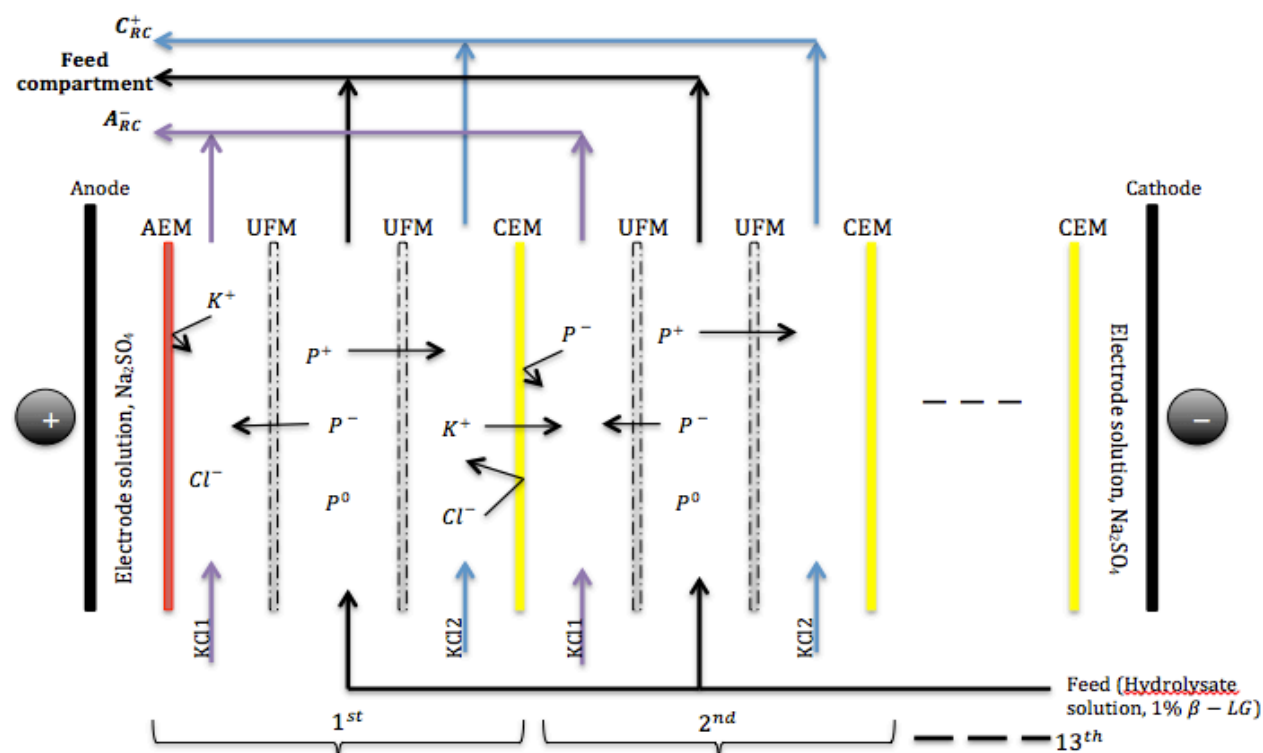


Figure 3.1 EDUF cell configuration for the simultaneous separation of anionic and cationic peptides. AEM: anion-exchange membrane, UFM: Ultrafiltration membrane, CEM: cation-exchange membrane, P^+ : cationic peptides, P^- : anionic peptides, P^0 : neutral peptides.

3.3 Protocols

3.3.1 Hydrolysis of BiPro

Hydrolysis of BiPro was carried out in two batches of 20 L. 250 g of BiPro were dissolved in 20L of distilled water maintaining pH 7.8 and left overnight at 4°C. The day after, enzymatic hydrolysis was initiated by adding 100 mL of trypsin solution (25 g/L) at controlled temperature of 37°C in a double jacket reservoir (Scanima a/s, Aalborg, Denmark) while adjusting pH at 7.8 by adding 1N NaOH throughout an experiment of 120 minutes. The trypsin solution was prepared by weighing 2.5 g of trypsin in 100 mL of milli-Q water (conductivity $\leq 18.2 \mu\text{S}/\text{cm}$). After 120 minutes of enzymatic hydrolysis, tryptic action was inactivated by rising the temperature to 80°C for 30 minutes. Then the final hydrolysate was freeze-dried and stored at 4°C.

3.3.2 Preliminary tests

Preliminary tests were carried out with three cases to optimize the operational parameters. An overview of the preliminary tests is outlined in Table 3.2. The first two cases were done without applying any voltage in order to observe the effect of flow rate with pressure at different temperature in EDUF cell. *Case I*: All compartments were filled with de-ionized water. *Case II*: An aqueous KCl solution (2 g/L, 3 L) was taken in both feed and permeate compartments and Na₂SO₄ (20 g/L, 3L) as an electrode rinsing solution. Both cases were performed at three different temperatures (37°C, 27°C and 17°C) and the corresponding pressure was noted with increasing flow rate from 0.75 L/min to 3 L/min. *Case III*: A limiting current density was determined according to the method described by Cowan and Brown [62] and it was found to be 0.43 A/m². This preliminary test was done with 10 g/L of BiPro hydrolysate solution (3 L), 2 g/L KCL solution (3 L) and 20 g/L Na₂SO₄ aqueous solution (3L) in feed, peptides recovery and electrolyte compartment respectively. The initial pH of feed solution was manually fixed to 6 by addition of HCl (0.1 N) and a constant electric field of 0.7 V/cm was applied between the two electrodes. The pressure of 10 psi was maintained in recovery and feed compartments to avoid transmembrane pressure in the cell. The experiment was run at room temperature and changes afterward were recorded. 1.5 mL samples were collected from the feed and recovery compartments before applying the voltage and every ten minutes during the experiment of one hour. Current intensity, pH, conductivity and temperature were recorded every ten minutes all along the treatment. The conductivity and pH were controlled by KCl solution (100 g/L) and NaOH solution (1 N) or HCl solution (0.1 N), respectively. A constant electrode solution flow rate of 4 L/min and pressure of 4 psi was kept for all preliminary cases. Three replicates of each experimental condition were performed.

Table 3.2 Preliminary tests done with different test solutions at various experimental conditions.

Cases	Test Solution in different compartments			Experimental conditions	
	<i>Feed</i>	<i>Recovery</i>	<i>Electrolyte</i>	<i>Voltage</i>	<i>Temperature</i>
I	DI water	DI water	DI water	NA	17, 27 & 37°C (3 replicates each)
II	KCl solution (2 g/L, 3L)	KCl solution (2 g/L, 3L)	Na ₂ SO ₄ solution (20 g/L, 3L)	NA	17, 27 & 37°C (3 replicates each)
III	Hydrolysate (10 g/L, 3L)	KCl solution (2 g/L, 3L)	Na ₂ SO ₄ solution (20 g/L, 3L)	10 Volts	Room temperature=27°C (3 replicates)

DI water: de-ionized water, NA: not applied

3.3.3 Electroseparation procedure

Three different experimental conditions were assessed namely, Direct Current (DC), Pulsed Electric Field (PEF) and Reverse Polarity (RP) to study the effect on migration rates of anionic and cationic peptides in EUR6 cell. The procedure for the three conditions were followed as described in abovementioned *case III* of preliminary tests except the conductivity of feed solution which was made equal to the permeate compartment by adding 3 g of NaCl solution. A calibration curve of NaCl conductivity as a function of concentration was plotted (shown in Figure 3.2). In this condition the limiting current density was found to be $0.553 \pm 0.026 \text{ A/m}^2$. EDUF was carried out by using constant voltage of 10 V using a Xantrex power supply (Model HPD 60-5SX; Burnaby, BC, Canada). A pulse/pause of 2s/0.2s (ratio=10) was applied for PEF and a current application duration of 2s and reverse polarity (RP) of 0.2s was applied for RP. The RP and PEF generation was generated using a one mode generator (Pulsewave 760, Bio-rad, USA). Three replicates of each condition were performed. Membrane cleaning was done with demineralized water between two replicates while complete acid-base-acid cleaning was performed before starting third replicate.

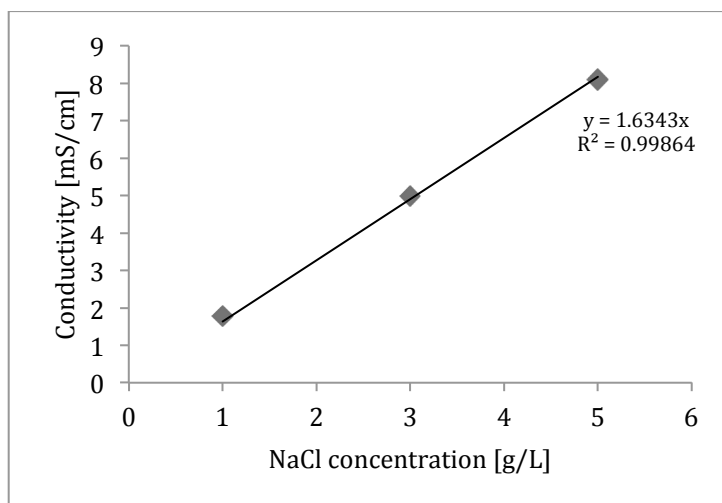


Figure 3.2 Calibration curve of conductivity as a function of NaCl concentration at room temperature.

3.4 Analysis method

3.4.1 Solution conductivity

The conductivity of feed and permeates (A_{RC}^- and C_{RC}^+) was measured using a YSI conductivity meter (Model 3100) equipped with an automatic temperature compensation (ATC) YSI immersion probe model 3252, cell constant $K = 1 \text{ cm}^{-1}$ (Yellow Springs Inc, OH, USA).

3.4.2 pH

A pH-meter model SP20 (Thermo Orion, West Chester, PA, USA) with a VWR Symphony epoxy gel combined pH electrode (Montréal, Canada) was used.

3.4.3 Total peptide concentration determination in liquid samples

The total peptide migration in A_{RC}^- and C_{RC}^+ was determined using BCATM protein assay (Pierce Biotechnology Inc., Rockford, IL, USA) for all conditions. Samples were prepared in 96 wells microplate (Corning Inc. NY, USA) and incubated at 37°C for 30 minutes and then cooled at room temperature. The absorbance was read at 562 nm on a microplate spectrophotometer (THERMOMax, Molecular devices, Sunnyvale, CA). The concentration of anionic and cationic peptides was determined with a standard calibration curve in a range of 25-2000 $\mu\text{g/mL}$ of bovine serum albumin (BSA).

3.4.4 Determination of total peptides in freeze dried sample

The initial protein concentration of BiPro before and after hydrolysis was determined by the total nitrogen determination in the samples. Total nitrogen content in the sample was analyzed by combustion of 150 mg sample using a LECO-FP528 carbon and nitrogen analyzer (LECO, St. Joseph, Michigan, USA). Nitrogen content in the samples were converted into protein percentages by multiplying with a conversion factor of 6.38 that is the value commonly used for milk proteins [63].

3.4.5 Protein and peptide profiles

The peptide composition of the initial hydrolysate, A_{RC}^- and C_{RC}^+ was determined by RP-HPLC according to the method of Firdaous et al. [53] which is adapted to the specific conditions of the β -Lg and peptides generated during enzymatic hydrolysis. A sample for analysis was prepared in a 2 ml vial with 0.2 μm PVDF (Canadian Life Science, ON, Canada) filter before passing into system. The system used was an Agilent 1100 series. Peptides were analyzed with a Luna 5 μm C₁₈ column (2 i.d. \times 250 mm, Phenomenex, Torrance, CA, USA). Trifluoroacetic acid (TFA) 0.1% (v/v) in water as solvent A and acetonitrile/water/TFA (90%/ 10%/ 0.1% (v/v)) as solvent B were used for elution at a flow rate of 0.2 mL/min. A linear gradient of solvent B, from 3% to 55% in 85 min was used. Peptide elution was monitored at a wavelength of 214 nm [53], [64].

CHAPTER 4

RESULTS AND DISCUSSION

4. Results and Discussion

4.1 Preliminary tests

4.1.1 Pressure as a function of the flow rate (Case I and Case II)

As mentioned in Table 3.2, different solutions were tested to verify the relationship between flow rate and pressure in each compartment (Figure 4.1b) of the cell as a function of temperature. Despite of test solutions used, a linear relation between the pressure and flow rate was observed in each compartments at different temperatures. It is observed that the pressure obtained for the respective flow rates in each compartments were slightly higher when test was done with water than KCl solution; 9.56 versus 8.70 psi/L.min⁻¹, 6.84 versus 6.04 psi/L.min⁻¹ and 5.75 versus 5.56 psi/L.min⁻¹ for feed, A_{RC}^- and C_{RC}^+ compartments respectively. A test done by either solutions (water or KCl) had higher slope in feed compartment than recovery compartments (slope=9.56 with respect to 6.84 and 5.75 for water and slope=8.70 with respect to 6.04 and 5.56 for KCl) which means a respective pressure can be achieved at lower flow rate in feed compartment than recovery compartments. It is also seen that the slope is quite similar (5.75 for water and 5.56 for KCl) in C_{RC}^+ for both case. During the test it was noted that the flow rate in feed compartment cannot be increased after attaining certain maximum value; 19 psi at 2.2 L/min for water and 16 psi at 1.8 L/min for KCl.

It was also noticed that there was not a significant effect of temperature in the relation of pressure and flow rate in each compartment. However, the different pressures were observed for the same flow rate in the feed and recovery compartments. At flow rate of 2 L/min, a pressure of 17.5, 12 and 9.6 psi was observed in the case of water solution for feed, A_{RC}^- and C_{RC}^+ compartments respectively. Similarly, in the case of KCl solution, a pressure of 14, 9.5 and 7.5 psi was found at 1.5 L/min for feed, A_{RC}^- and C_{RC}^+ compartments respectively. The variation in compartmental pressure can be explained due to the presence of different membranes in the configuration and their tendency of loss of charge. Feed stream passes through two ultrafiltration membranes. Ultrafiltration membranes used were asymmetric membrane (as shown in Figure 4.1a). The filtering side of UFM is smoother than non-filtering side and it is assumed that the presence of roughness in non-filtering side affects the flow tendency along the membrane (Suwal et al, 2014). Consequently, this leads to a loss of charge and impact the flow-pressure relation in the compartments. The difference in compartmental pressure may also be due to the compactness of membranes, that is uneven inter-spacing among the membranes.

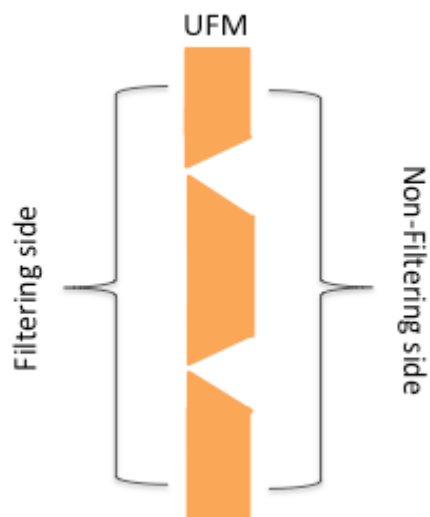


Figure 4.1a A schematic representation of non-symmetric UFM.

Furthermore, it was preferred to maintain the same pressure in feed and recovery compartments to avoid the formation of a transmembrane pressure (TMP). Therefore, the mean pressure of 10 psi was chosen to avoid bubble formation inside the tube of solution flow (in case of lower pressure) and to minimize the external leakage from the cell (in the presence of higher pressure). A room temperature was chosen considering no significant effect of temperature in pressure and flow relation which can also minimize the energy cost. The flow rates required to maintain the pressure of 10 psi at 27°C (room temperature) in feed, A_{RC}^- and C_{RC}^+ compartments were found to be 1.08 ± 0.02 , 1.63 ± 0.10 and 2.00 ± 0.05 $L.min^{-1}$ respectively.

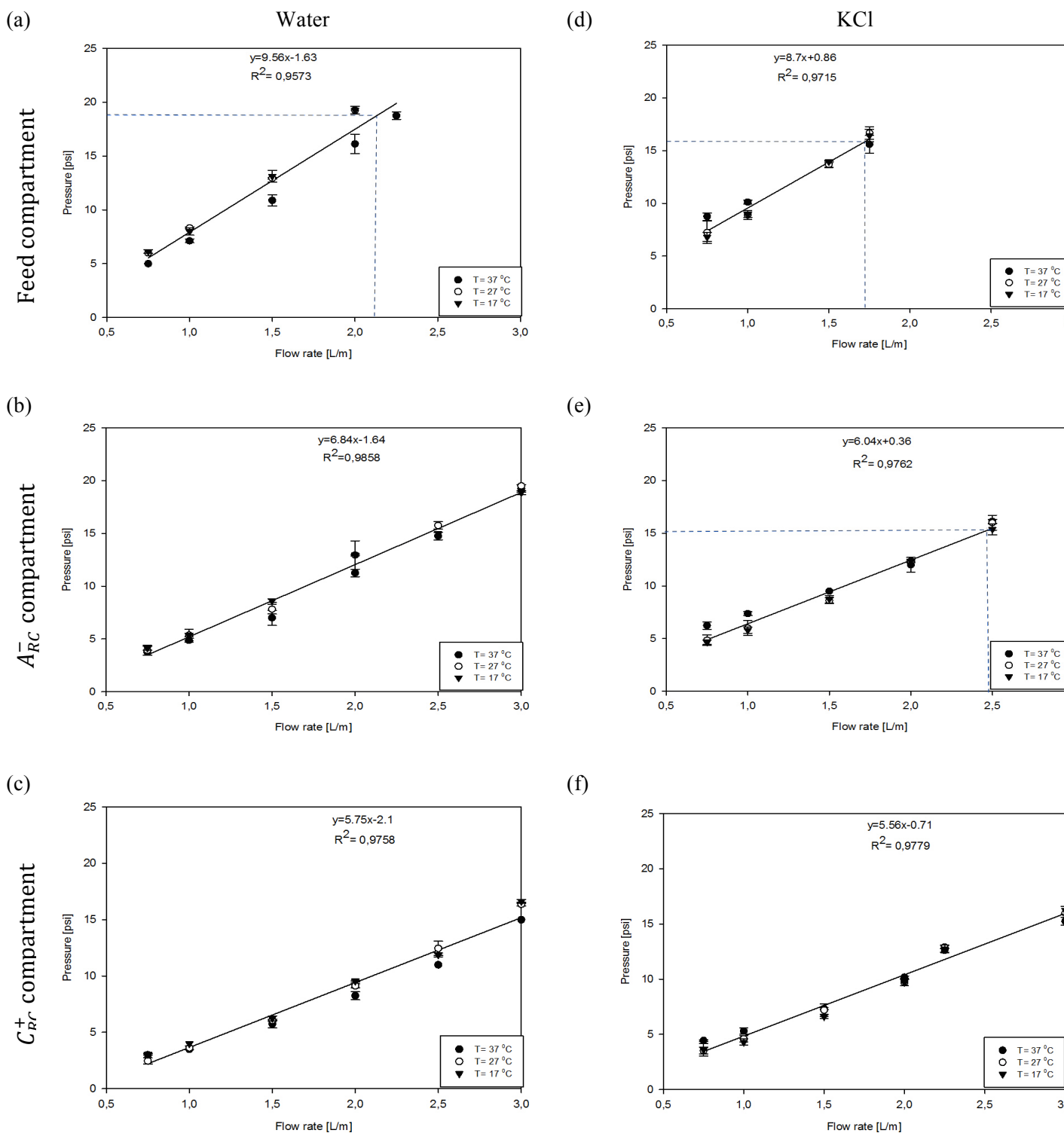


Figure 4.1b Evolution of pressure as a function of flow rate in feed compartment (a,d), A_{RC}^- (b, e) and C_{RC}^+ (c, f) done only with de-ionized water (a, b, c) and KCl solution (d, e, f) at temperature, $T = 37^\circ\text{C}$, 27°C and 17°C . A_{RC}^- : anionic peptides recovery compartment, C_{RC}^+ : cationic peptides recovery compartment.

4.1.2 Preliminary tests to study electrodynamic parameters (Case III)

The section below described the preliminary experiments carried out with BiPro hydrolysate as feed solution. A constant pressure of 10 psi was maintained by adjusting the respective flow rates for different compartments as discussed in the previous section (4.1.1).

4.1.2.1 Limiting current density

I-V curve (Figure 4.2) was used to find the limiting current density for these conditions of experiment. The limiting current density was found at 12 V. The experiment was carried out under the limiting current density at 10 V to avoid water-splitting which has a consequence in energy loss due to water splitting rather than ion transport. In addition, water splitting can change in pH of respective compartment, increase in membrane resistance and change in membrane properties, for instance, OH^- can degrade AEM.

A constant pH (data not shown) observed in the feed compartment during the experiment also revealed that the applied current was smaller than limiting current density [65].

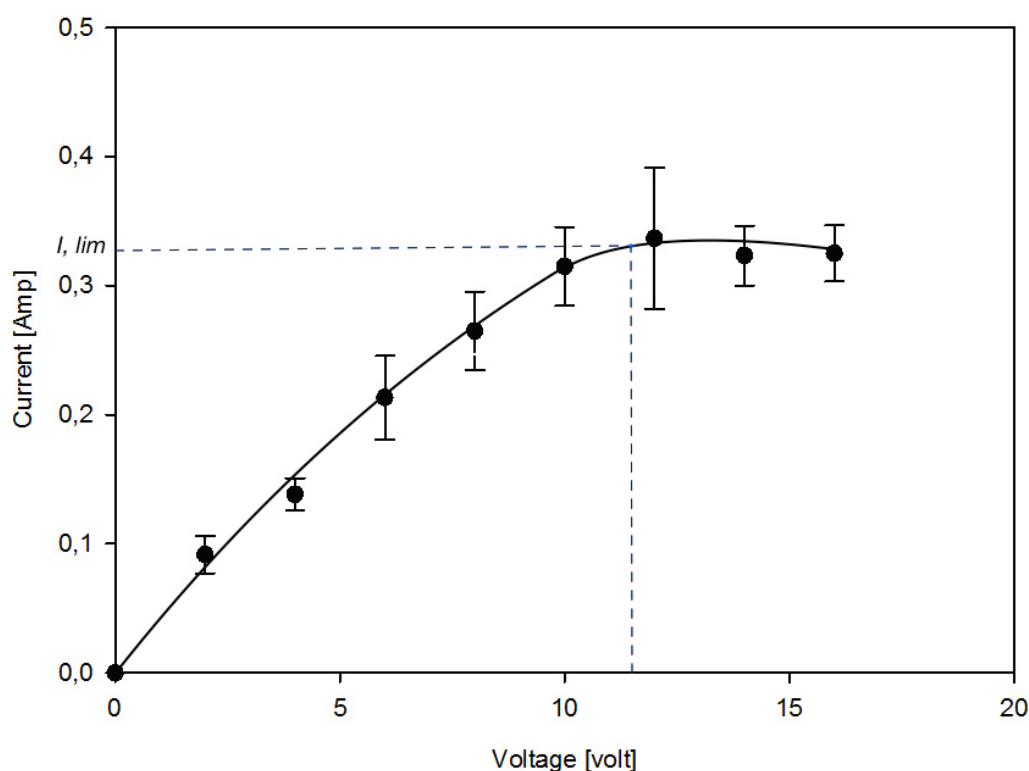


Figure 4.2 I-V curve for determining limiting current density.

4.1.2.2 Evolution of current intensity

Figure 4.3 showed the current intensity increased linearly ($R^2=0.9979$), from initial average value of 0.23 ± 0.02 A to final average value of 0.33 ± 0.01 A, during the experimental period of 60 minutes.

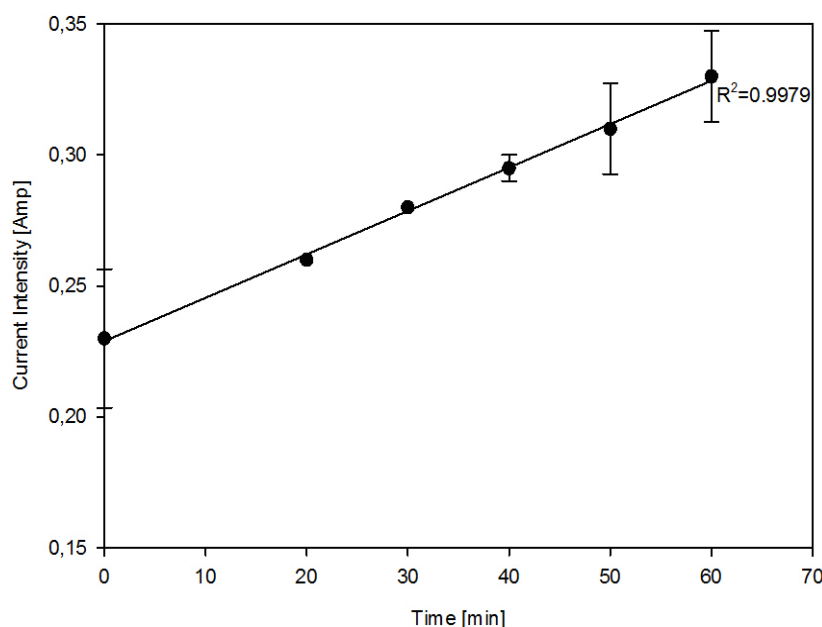


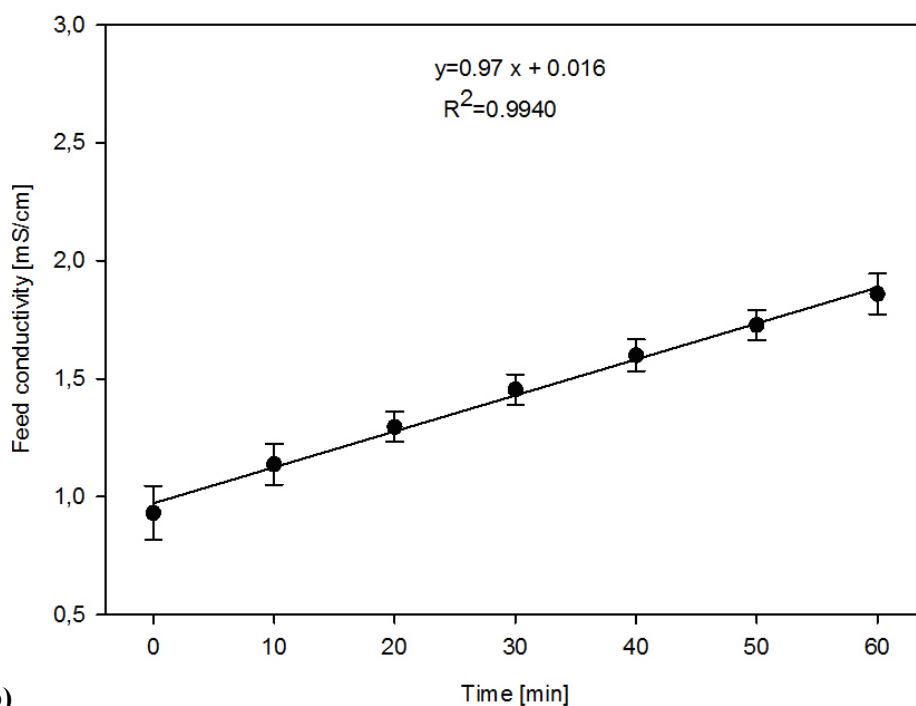
Figure 4.3 Change in current intensity with time during experiment of 60 minute at pH 6 and $T=27^\circ\text{C}$.

4.1.2.3 Solution electrical conductivity

Figure 4.4 represents the changes in solution electrical conductivity in feed (Figure 4.4a) and A_{RC}^- compartments (Figure 4.4b) during the 60 minutes of EDUF treatment. The solution conductivity increased linearly in feed ($R^2=0.994$) and A_{RC}^- ($R^2=0.951$) compartments: conductivities increased significantly as a function of time from 0.93 ± 0.11 to 1.85 ± 0.08 mS/cm (slope= 0.97) and from 2.49 ± 0.16 to 3.00 ± 0.13 mS/cm (slope= 2.43), respectively, while it decreased in C_{RC}^+ (data not shown) soon after the voltage was applied to EDUF cell. The conductivity in C_{RC}^+ was maintained constant at initial value of 2.33 ± 0.05 mS/cm by adding KCl solution (100 g/L) as explained by Suwal et al.[66] since it does not allow increase in local resistance in the cell. In contrast, Poulin et al.[8] has found that the conductivity of A_{RC}^- decreased over time, regardless the number of stacks used. Unlike the current configuration, Poulin et al. [67] had used one UFM (20 kDa) sandwiched between two IEMs that resulted in demineralization of the A_{RC}^- compartment at pH 5. Doyen et al.[68] also reported the decrease in A_{RC}^- conductivity using two UFM (50 kDa) sandwiched between two IEMs at pH 7.8. Though the electrodialytic parameters were different between present and previous studies but the difference in conductivities of feed and recovery compartment mainly can be described with respect to cell stacking. In this study, the configuration of EDUF cell with 13 repetitions of the stack was used while the previous study was made with one single unit. Figure 6 also showed that the conductivity in feed increased by two-fold compared to A_{RC}^- than their respective initial value. This can be explained since two ions (K^+

and Cl^-) migrated to feed compartment from A_{RC}^- and C_{RC}^+ compartments respectively, while only Cl^- ion remains in A_{RC}^- since it could not pass through the CEM resulting in its accumulation and hence to an increase in conductivity. This is also the reason of the increase in current intensity (Figure 4.3) with time during EDUF treatment.

(a)



(b)

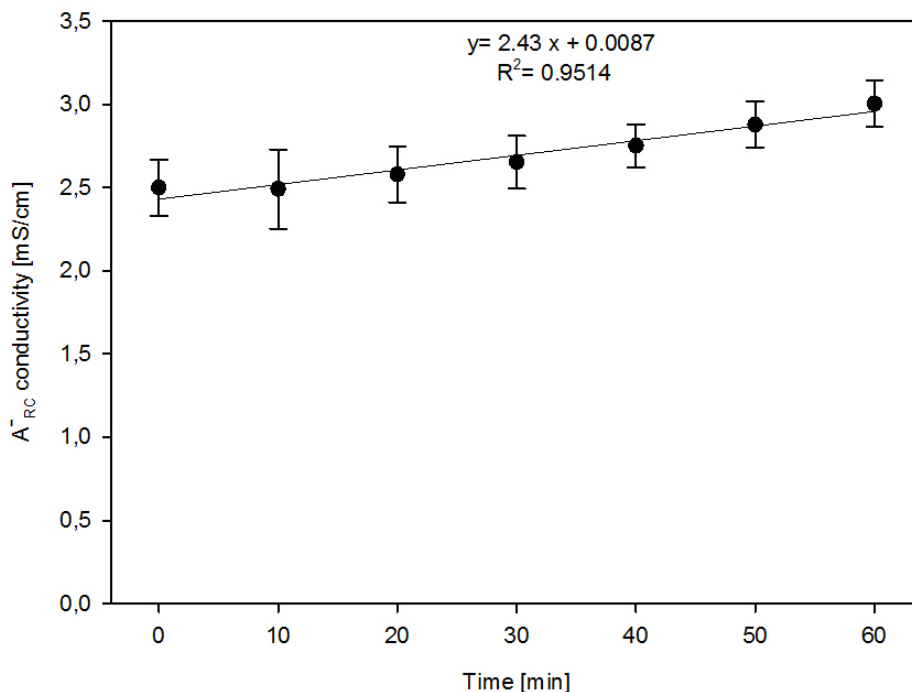


Figure 4.4 Evolution of conductivity in feed (a) and A_{RC}^- compartment (b) during 60 min-EDUF treatment at pH 6, constant voltage of 10 V and at room temperature.

4.1.2.4 Evolution of peptide concentrations in A_{RC}^- and C_{RC}^+ recovery compartments

Figure 4.5 represents the concentration of peptides in A_{RC}^- and C_{RC}^+ compartments. The peptides concentration in both compartment increased during the treatment of 60 minutes. However the final concentration of peptides in A_{RC}^- is two-fold higher than in C_{RC}^+ with values of $196.7 \pm 86.7 \mu\text{g/mL}$ and $84.8 \pm 26.9 \mu\text{g/mL}$ respectively which correspond to peptide migration rates of $0.57 \pm 0.25 \text{ g/m}^2\text{h}$ and $0.29 \pm 0.09 \text{ g/m}^2\text{h}$. The difference can be explained by the fact that a tryptic hydrolysis of β -Lg generates mostly anionic peptides at pH 7.8 [68][69][70] which induces rapid and higher migration rates. Furthermore, the membrane and peptides interaction can also influence the migration rate of peptides. Since the charge of the peptides and membrane surface varies according to the experimental pH. Following these results, a further experimental design was developed to study the effect of electric field conditions, DC, PEF and RP on EDUF peptide migration rates.

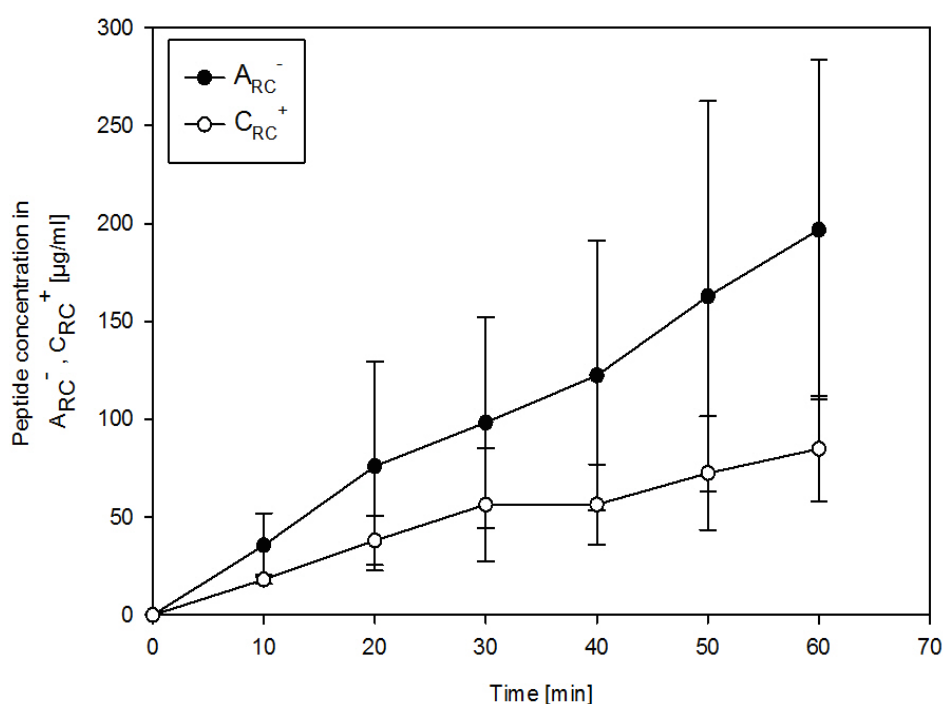


Figure 4.5 Concentration of anionic and cationic peptides as a function of time at pH 6, constant voltage of 10 V and at room temperature.

4.2 Effect of application of PEF and RP conditions on peptide migration and selectivity

4.2.1 I-V curve

Figure 4.6 shows the current intensity as a function of voltage before performing an experiment under the conditions of DC, PEF and RP. The limiting current density is observed once again at 10 V. It is due to the presence of electric field as a driving force that reduced the possibility of deposition of uncharged peptides on UFM's unlike the pressure driven processes [8]. Hence, it theoretically means that there was no membrane fouling. As a result, the limiting current density was unchanged even after three repetitions of each condition were carried out.

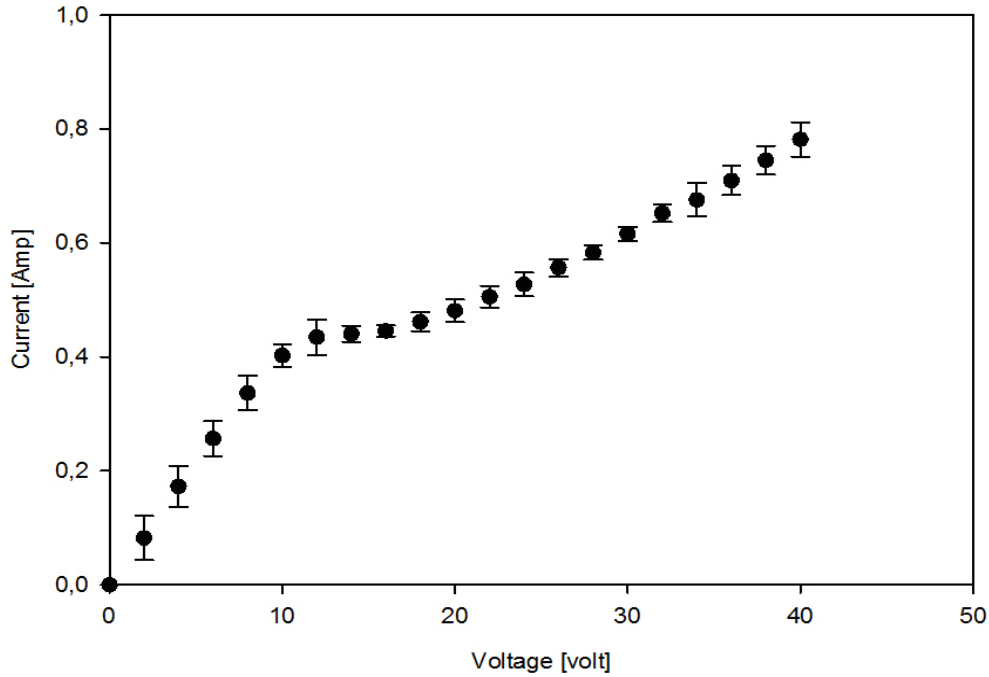
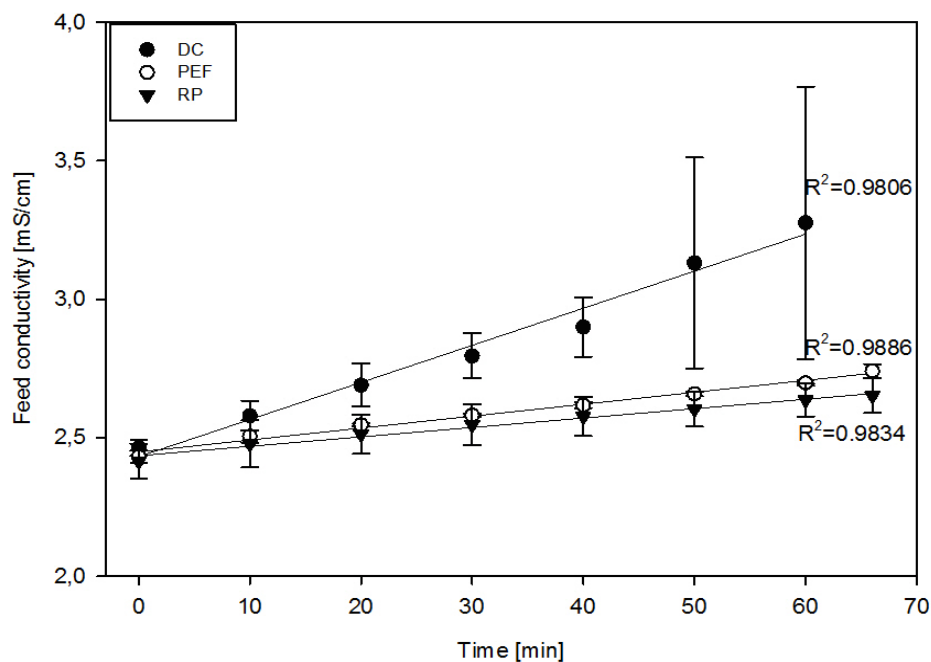


Figure 4.6 I-V curve before performing DC, PEF and RP experimental conditions. DC: direct current, PEF: pulsed electric field, RP: reverse polarity.

4.2.2 Evolution of conductivities

Whatever the experimental conditions, the conductivity in feed solution increased linearly with time as shown in Figure 4.7a. 3 g of NaCl was added in feed compartment before starting the experiments in order to increase the initial feed conductivity. Addition of salt in feed also increased the initial current intensity due to the global distribution of conductivity through the feed channels and hence to maintain the constant electric field. As the treatment proceeded the conductivity in feed compartment linearly increased during the 60 min treatment under DC ($R^2=0.9806$), PEF ($R^2=0.9886$) and RP ($R^2=0.9834$) conditions. The increase in feed conductivity can be explained as previously by K^+ and Cl^- migration into the feed compartment from A_{RC}^- and C_{RC}^+ compartments, respectively. The same tendency had been found by Doyen et al. [68] even when a single unit was used. Nevertheless, the final feed conductivities for DC, PEF and RP were 3.27 ± 0.49 , 2.74 ± 0.02 and 2.65 ± 0.06 mS/cm respectively, but the conductivity is half time higher in DC than PEF and RP. The reason of slower increase in conductivity is due to the pulse and pause effect generated by PEF and reversing the polarity in RP condition. Also, it can be seen in Figure 4.7a where the linear plot of PEF and RP are not so apart with no such significant changes through the treatment period. As a result the movement of ions from recovery compartment to feed compartment are controlled in the case of PEF and RP.

(a)



(b)

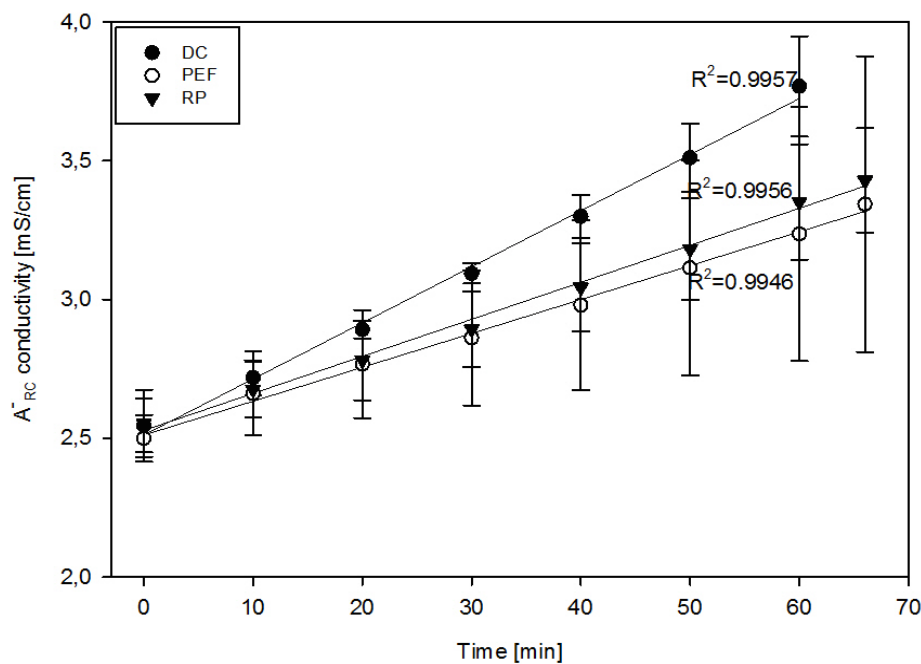


Figure 4.7 Evolution of conductivity in feed (a) and A_{RC}^- compartment (b) under DC, PEF and RP experimental conditions at pH 6. A_{RC}^- : anionic peptides recovery compartment, DC: direct current, PEF: pulsed electric field, RP: reverse polarity.

The change in conductivity in anionic recovery compartment during the treatment of 60 min is shown in Figure 4.7b. The conductivity in anionic recovery compartment increased rapidly compared to feed compartment. Likewise the conductivity in feed compartment, the final

conductivity under DC current is higher than PEF and RP in anionic recovery compartment. The final conductivity under DC, PEF and RP conditions are reported as 3.76 ± 0.17 , 3.34 ± 0.53 and 3.42 ± 0.18 mS/cm, respectively. The rapid increase in conductivity in anionic recovery compartment can be explained by obstruction of movement of Cl^- ion from the feed compartment towards the counter electrodes but the presence of CEM on pathway retained the Cl^- in anionic recovery compartment. However the PEF and RP conditions have similar linear trend due to the effect of electric field.

4.2.3 Total peptide migration in anionic and cationic compartments

The electrodynamic conditions have affected the final concentration of peptides in anionic and cationic peptide recovery compartments resulting in difference in migration rates (Figure 4.8 and 4.9).

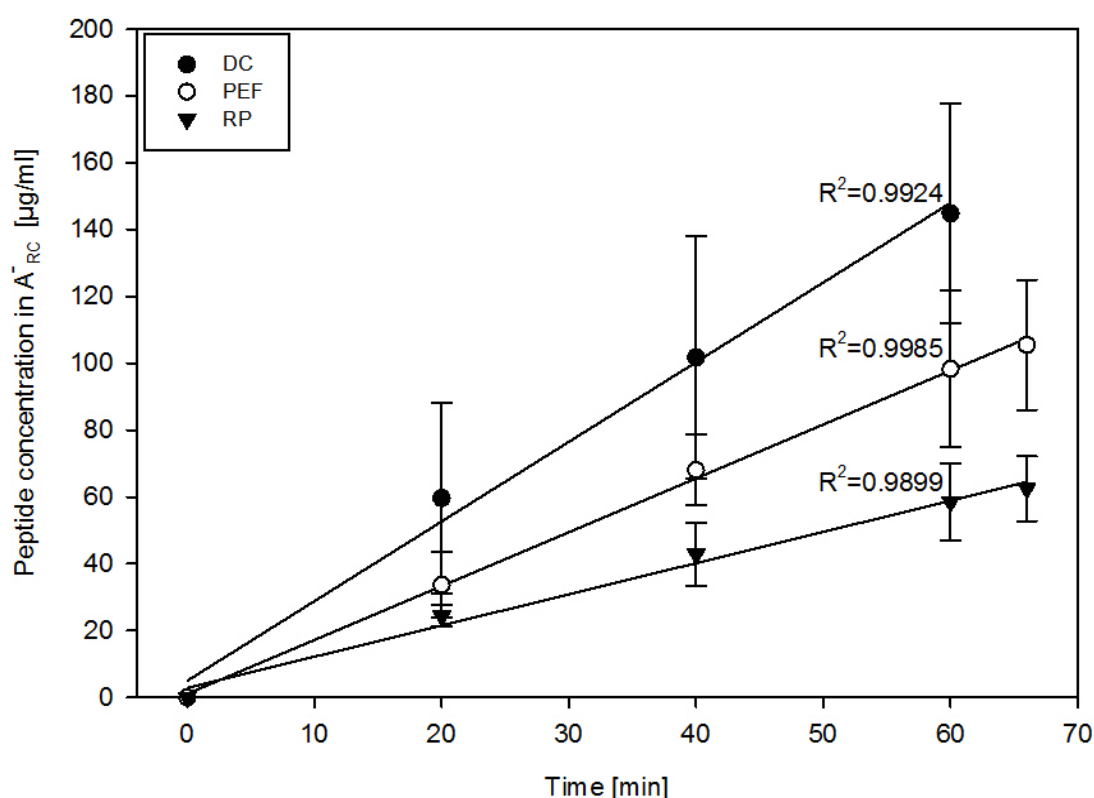


Figure 4.8 Evolution of peptides concentration in the A_{RC}^- under PEF, DC and RP conditions during EDUF treatment at pH 6. A_{RC}^- : anionic peptide recovery compartment, DC: direct current, PEF: pulsed electric field, RP: reverse polarity.

A linear trend of peptides concentration in A_{RC}^- was attained during the EDUF treatment. It was observed that peptides migration under DC condition is relatively higher than PEF and RP condition as similar to the trend of conductivity in anionic compartment (Figure 4.7b). The migration rate of peptides in anionic recovery compartment under DC, PEF and RP condition is presented in Table 4.1. Though the peptides migration rate is larger under DC, but PEF and RP showed lower standard deviation. The concentration of peptide in cationic

recovery compartment (C_{RC}^+) also increased linearly (Figure 4.9) during the EDUF process. The peptide concentration in C_{RC}^+ under all conditions is almost similar.

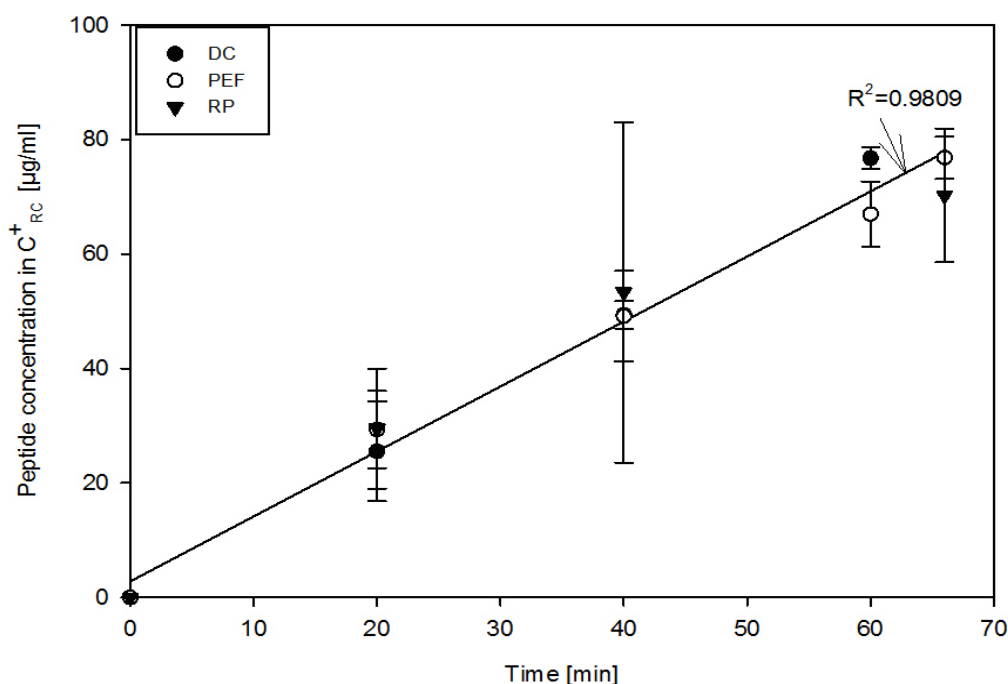


Figure 4.9 Evolution of peptides concentration in the C_{RC}^+ under PEF, DC and RP conditions during EDUF treatment at pH 6. C_{RC}^+ : cationic peptide recovery compartment, DC: direct current, PEF: pulsed electric field, RP: reverse polarity

It is also clear from Table 4.1 that the migration rates in cationic recovery compartment under DC, PEF and RP are almost the same. However, the total migration rate under DC condition is higher than PEF and RP with values of 0.69 ± 0.09 , 0.51 ± 0.05 and 0.38 ± 0.15 g/m².h respectively. This migration is smaller compared to the literature and work performed previously using the EDUF process where Poulin et al. [67] has recorded migration rate of 3.1, 6.87 and 11.23 g/m².h for electric field strength of 2.75, 5.5 and 11 V/cm respectively. The difference is explained by lower electric field strength used in the present study which was 0.7 V/cm. Yet the ratio of migration rate to electric field strength is comparable with the previous study in case of continuous current mode. Besides lower electric field strength, the cell configuration and MWCO of UFM (100 kDa) with effective surface area of 0.734 m² used in this study were different compared to 20 kDa of UFM membrane with effective surface area of 0.001 m² in the previous study. The main reason of lower migration rate can be explained by the lower electric field strength as from the previous result it is also justified that higher electric field resulted in higher migration rate of peptides. In addition, it is noted that overall migration rate in PEF and RP is comparatively lower than in DC. It is possibly due to the condition of pulse and pause implemented in PEF which impedes the continuous flow of peptides toward oppositely charged electrodes by cutting the electric potential field in every 2 seconds. In the case of RP, the polarity was altered at every 2 seconds which could possibly induce the backward flow of peptides known as retromigration from respective

recovery compartment to feed compartment resulting in lowest migration rate of peptides among all three conditions (Suwal et al.2015 thesis).

Table 4.1 Peptide migration rates in A_{RC}^- and C_{RC}^+ recovery compartment.

Recovery compartment	Migration rate (g/m ² .h)		
	DC	PEF	RP
A_{RC}^-	0.44 ± 0.10	0.28 ± 0.05	0.16 ± 0.02
C_{RC}^+	0.25 ± 0.00	0.23 ± 0.01	0.23 ± 0.03
Total	0.69 ± 0.09	0.51 ± 0.05	0.38 ± 0.15

A_{RC}^- : anionic peptides recovery compartment

C_{RC}^+ : cationic peptides recovery compartment

4.3 Peptide profiles

4.3.1 Peptides profile & molecular weight of BiPro hydrolysate

Figure 4.10 represents HPLC chromatogram of BiPro before enzymatic hydrolysis. The two major peaks ‘a’ and ‘b’ observed at retention times of 78.30 and 79.89 min respectively corresponds to $\beta - Lg$ A and $\beta - Lg$ B, the two major variants of $\beta - Lg$ protein in milk with respective molecular masses of 18,362.86 Da and 18,276.73 Da [71]. The peak detected at retention time of 73 min by HPLC may probably correspond to another milk protein, such as α -lactalbumin or bovine serum albumin (BSA) since the purity of BiPro was about 90% according to the manufacturer (DAVISCO).

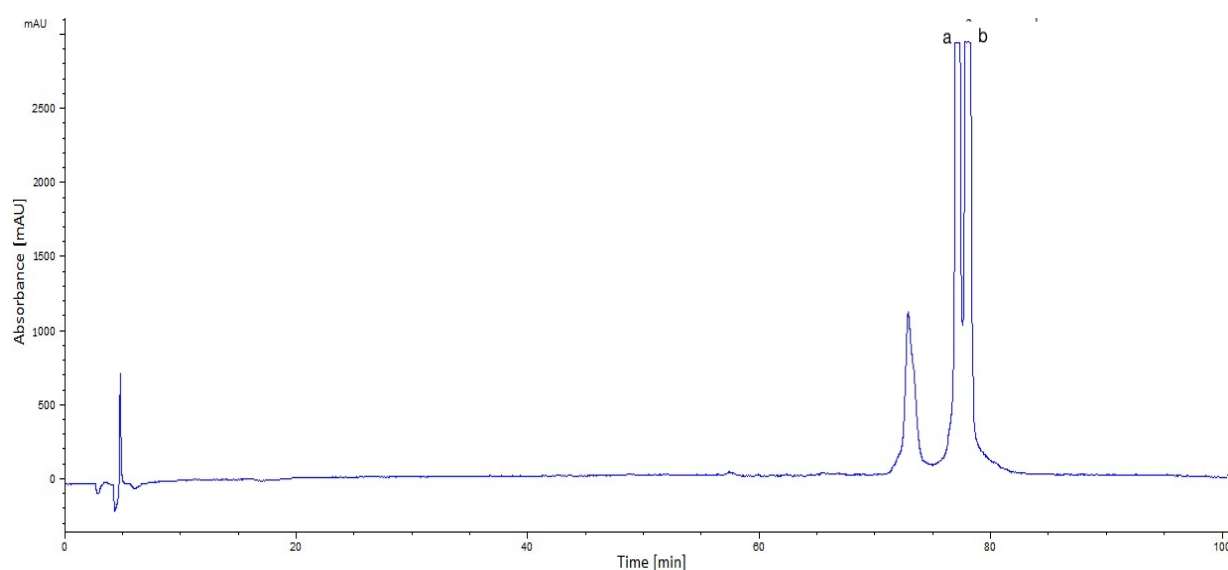


Figure 4.10 Chromatogram of initial BiPro (before enzymatic hydrolysis).

Figure 4.11 illustrates chromatogram of BiPro hydrolysate. BiPro was completely hydrolyzed since no peak from native protein is observed when compared with figure 4.10. HPLC analysis showed that hydrolysis of BiPro gave 27 major peaks. Furthermore the molecular masses of the 27 peaks containing peptides were characterized by MS. Potential peptide sequences, location of sequences, net charge and isoelectric point (pI) presented in Table 2 were obtained by using tools available on the ExPASy Bioinformatics Resource Portal (Swiss Institute of Bioinformatics). A total of 31 peptides were obtained from theoretical tryptic hydrolysis of BiPro, where 19 peptides with MW ranging from 572.9 Da to 1249.5 Da were derived directly from enzymatic hydrolysis of β – Lg protein. The sequence of 6 peptides found to be generated from tryptic hydrolysis of BSA and 6 peptides sequences marked as “nd” in Table 4.2 were seemingly not generated from tryptic hydrolysis but supposed to be formed due to peptide-peptide interactions or from other protein source, which was already observed by Doyen et al. [68], [72].

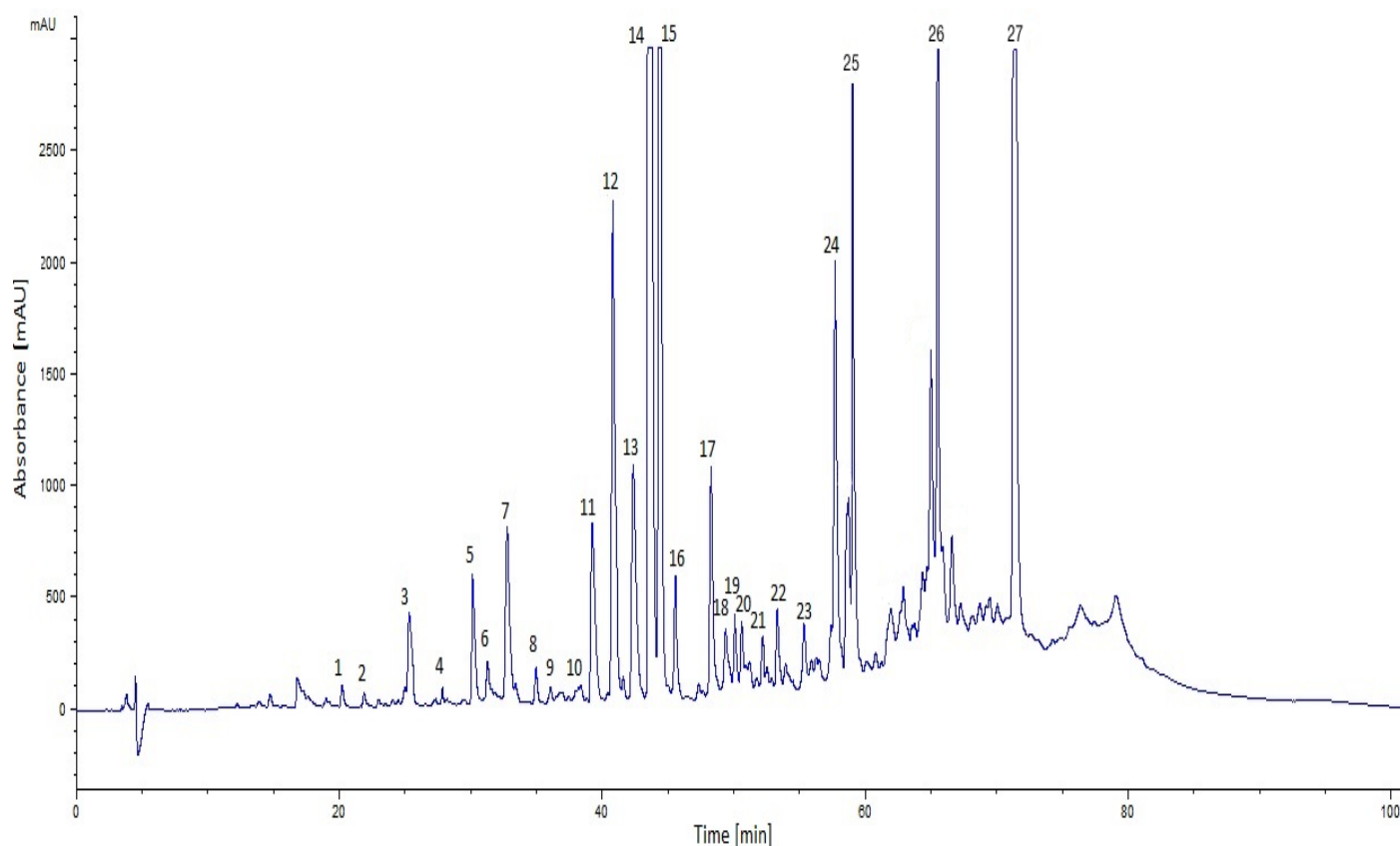


Figure 4.11 Chromatogram of BiPro hydrolysate after enzymatic hydrolysis.

Table 4.2 Characterization of peptides obtained after 120 min of tryptic hydrolysis of BiPro.

Peak#	R _t (min) ^a	Obs MW ^b	Potential sequence	Location ^c	Net charge ^d	pI ^e
1	22.3	617.9	EQLTK ^g	f1-05	nd	nd
2	24.3	804.7	IIAEKT	f71-77	nd	nd
3	25.5	572.9	IIAEK	f71-75	—	6.0
4	27.5	856.7	DAQSAPLR	F33-40	—	5.84
	28.1	700.9	DAQSAPL	f33-39	—	3.80
	29.6	915.7	IDALNENK	f84-91	—	4.37
5	30.4	915.7	nd ^f	nd	nd	nd
6	31.6	1043.6	KIDALNENK	f83-91	+	6.07
7	32.9	672.9	GLDIQK	f9-14	—	5.84
8	33.4	948.7	nd ^f	nd	nd	nd
9	35.2	1244.5	TPEVDDEALEK	f125-135	—	3.8
10	37.1	1256.5	VLAASSARQRLR ^g	f188-198	nd	nd
11	39.4	932.7	LIVTQTMK	f1-8	+	8.8
12	41.0	836.8	ALPMHIR	f142-148	+	9.8
13	42.5	1192.6	VLVLDTDYKK	f92-101	—	5.93
14	43.8	902.8	TKIPAVFK	f76-83	+	10.0
15	44.6	695.7	VAGTWY	f15-20	—	5.49
16	45.7	1064.6	WENGECQK	f61-69	—	4.5
17	47.5	1007.7	nd ^f	nd	nd	nd
18	48.5	2903.5	DVCKNYQEAKDAFLGSFLYE YSRR ^g	f313-336	nd	nd
19	49.5	1162.6	LVNELTEFAK ^g	f42-51	nd	nd
20	50.4	774.8	TKIPAVF	f76-82	+	8.41
21	51.0	1199.6	nd ^f	nd	nd	nd
22	52.9	1249.5	FKDLGEEHFK	F35-44	—	5.4
23	53.6	2306.9	nd ^f	nd	nd	nd
	54.7	1013.7	QTALVELLK ^g	f525-533	nd	nd
24	57.8	2028.3	LGEYGFQNALIVRYTRK ^g	f21-40	nd	nd
25	59.5	1156.6	nd ^f	nd	nd	nd
	61.0	1190.5	SLAMAASDISLL	f21-32	—	3.80
26	64.4	931.7	KIIAEKT	f70-71	nd	nd
27	71.4	2704.9	VAGTWYSLAMAASDISLLDAQSAPLR	f15-40	—	4.2

^a Retention time^b Observed molecular weight^c Peptide location on the mature β -Lg protein^d Calculated at pH 7.8^e Isoelectric point^f Not determined^g Peptides generated by hydrolysis of bovine serum albumin

4.3.2 Profiles of peptides recovered in anionic and cationic recovery compartments

4.3.2.1 Peptides profile obtained from preliminary test (case III) of EDUF treatment

A typical HPLC chromatogram of peptides fraction obtained in anionic recovery compartment after EDUF of 60 min is displayed in Figure 4.12. Among the 11 peaks identified in A_{RC}^- , 5 peaks (#11, 12, 14, 16, 26) were from the β -Lg source. According to the relative area of the peaks, peak #12 and 14 with peptide sequence ALPMHIR and TKIPAVFK, respectively appeared higher in area than other peaks.

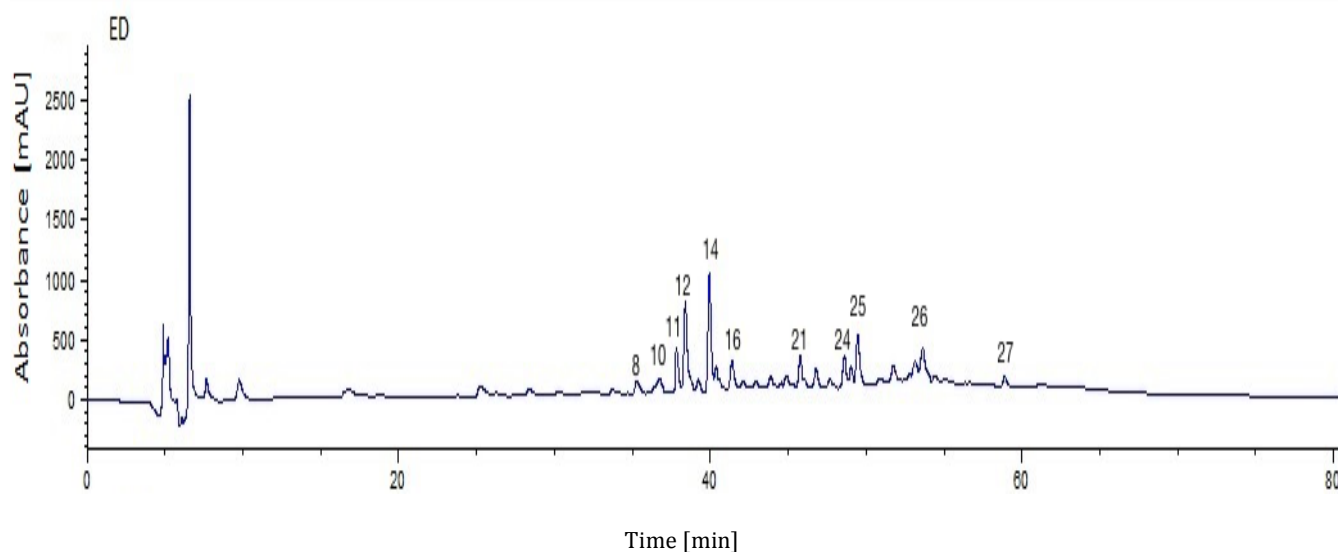


Figure 4.12 Chromatogram of peptides obtained in anionic peptide recovery compartment after 60 min of EDUF treatment.

In the chromatogram of peptides fraction in C_{RC}^+ (shown in Figure 4.13), 7 peaks were identified. It is observed that peak #12 and 14 have markedly migrated in cationic peptides recovery compartment than anionic peptides recovery compartment. It might be due to the fact of charge of those peptides. Since ALPMHIR and TKIPAVFK were positively charged at pH 6 (pI 9.8 for peak #12 and pI 10 for peak #14) which as a result migrated towards cationic peptides recovery compartment and hence are larger in quantities compared to that found in A_{RC}^- . A markedly migration of peak #12 and 14 could also be facilitated due to the negative charge of UFM's which as a result can easily allow to pass a positively charged peptide. Secondly, the molecular weight of these peptides (836.8 Da for peak #12 and 902.8 Da for peak #14) are lower which allows the cationic peptides to migrate towards C_{RC}^+ .

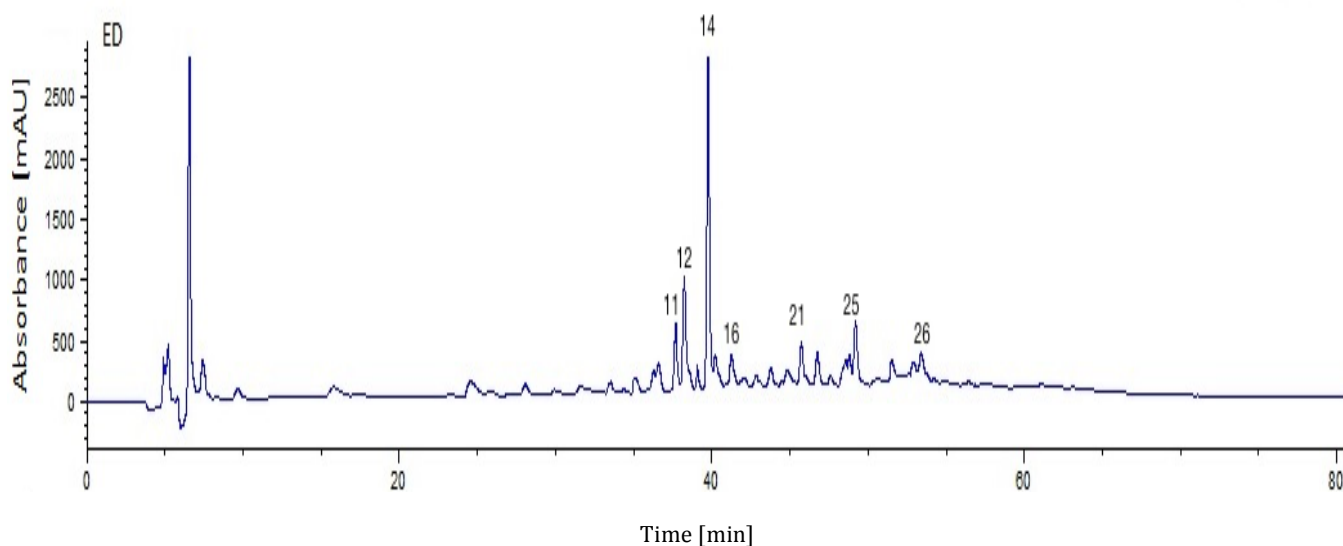


Figure 4.13 Chromatogram of peptides obtained in cationic peptide recovery compartment after 60 min of EDUF treatment.

4.3.2.2 Peptides profile obtained under the experimental conditions of DC, PEF and RP.

Figure 4.14 showed the HPLC chromatogram of peptides fraction recovered in the anionic peptides recovery compartment when treatment was done under DC, PEF and RP conditions. Under DC conditions, total of 12 peaks were identified and among which 8 peaks were found to be from the β -Lg source. It is observed that peaks #12, 14, 24, 25, 26 and 27 are relatively higher in area than other peaks.

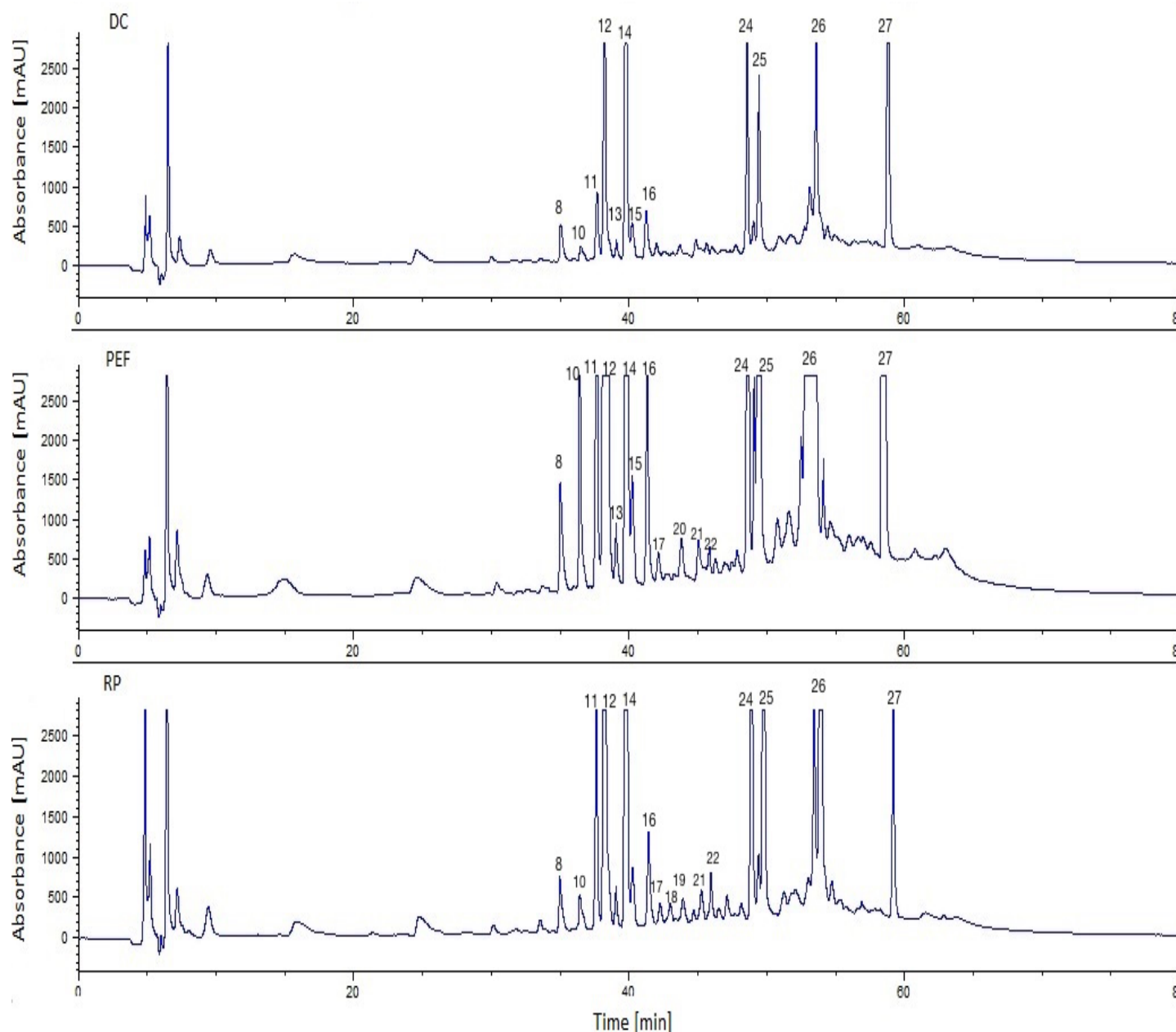


Figure 4.14 Chromatogram of peptides obtained in the A_{RC}^- under PEF, DC and RP conditions during EDUF treatment at pH 6. A_{RC}^- : anionic peptide recovery compartment, DC: direct current, PEF: pulsed electric field, RP: reverse polarity.

HPLC analyses performed on cationic peptides recovered in C_{RC}^+ after 60 min EDUF treatment at pH 6 under DC, PEF and RP conditions are shown in Figure 4.15. Under DC, PEF and RP conditions 14, 15 and 12 peaks were obtained respectively. Although the peaks originated from the β -Lg source were 9 peaks from DC, 7 peaks from PEF and 7 peaks from RP condition. If the number of peaks obtained is narrowed down to the peaks that corresponds to cationic peptides (positively charged peptides) then it is found that 4 peaks of cationic peptides sequence are obtained under DC while 3 peaks each are found under PEF

and RP conditions. It is also observed that 3 peaks of cationic peptides (peak #11, 12, 14) are found common whatever is the condition and their peptide sequences are LIVTQTMK, ALPMHIR and TKIPAVEK. It is due to the positive charge of these peptides at pH 6 (pI 8.8 for #11, pI 9.8 for #12 and pI 10 for #14) and also their relatively lower molecular mass 932.7, 836.8 and 902.8 Da respectively. However the peptide sequence GLDIQK that corresponds to peak # 7 is only noticed in the chromatograms under PEF condition. Besides this, peak #11, 12, 14, 16, 24, 26 and 27 under either condition relatively possessed larger peak area.

A comparison between the chromatograms of peptides obtained in A_{RC}^- and C_{RC}^+ compartment under DC, PEF and RP conditions showed almost the similar peaks and peptide sequences. Although it is found that few peaks under DC condition (peak #20, 21 and 22) in C_{RC}^+ are completely missing in A_{RC}^- . Similarly, peak #7 and #18 in C_{RC}^+ under PEF condition are missing from A_{RC}^- and peak #17 and #18 in A_{RC}^- under RP from C_{RC}^+ are missing. This can be explained due to the larger molecular mass and pI of these peptides.

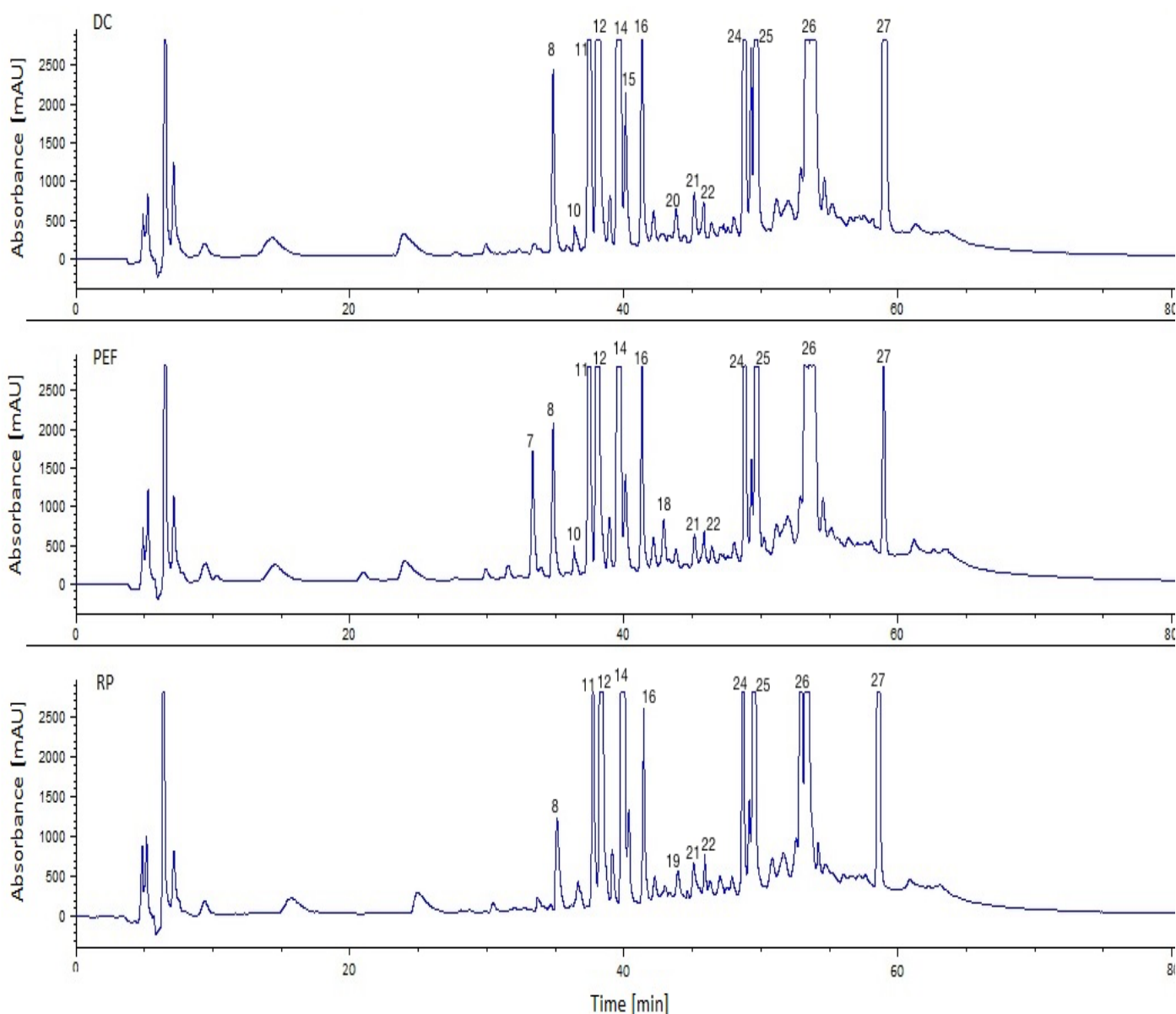


Figure 4.15 Chromatograms of peptides obtained in the C_{RC}^+ under PEF, DC and RP conditions during EDUF treatment at pH 6. C_{RC}^+ : cationic peptide recovery compartment, DC: direct current, PEF: pulsed electric field, RP: reverse polarity

However, the chromatograms of BiPro hydrolysate (shown in Figure 4.11) and peptides sequences presented in Table 4.2 showed the presence of bioactive peptides. Amongst 19 peaks identified from the source of $\beta - Lg$, 6 peptides had bioactive functionality. Peptide sequence IIAEK (peak #3) and GLDIQK (peak #7) were previously identified as hypocholestrolemic [39]. Peptide sequence IDALNENK (peak #4) and VAGTWYSLAMAASDISLLDAQSAPLR (peak #27) possess antihypertensive function [73] and also ALPMHIR (peak #12) was identified as antihypertensive [22]. It was also found that peptide sequence VLVLDTDYKK (peak #13) was marked as antibacterial [40].

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVE

5. Conclusions and Future Perspective

Enzymatic hydrolysis of BiPro has isolated peptides fractions, amongst these 6 were found to have bioactive peptides. Separation of bioactive peptides of specific interest (in this case peptide sequence ALPMHIR) was studied on pilot-scale EDUF (EUR6) model. Preliminary tests were done to establish the pressure flow relation in the different compartments. Later EDUF preliminary test investigated the current density at 10V and showed the relation of solution conductivity on migration of peptides in the recovery compartments (A_{RC}^- and C_{RC}^+). The peptides migration was found relatively higher in A_{RC}^- than C_{RC}^+ corresponding to values of $196.7 \pm 86.7 \mu g/mL$ and $84.8 \pm 26.9 \mu g/mL$ respectively due to higher concentration of anionic peptides generated by tryptic hydrolysis of bipro at pH 7.8 or possibly due to less peptide-membrane interaction. The study on the effect of three electric field conditions (DC, PEF and RP) showed the influence of electrodialytic parameters on migration rate. Under DC condition migration rate was relatively larger with a value of $0.69 \pm 0.09 g/m^2.h$, while migration rate dropped by 25% under PEF condition and approximately decreased to 50% under RP condition. It was also found that some of the peptides missing under DC were observed during PEF and RP condition. This showed that PEF and RP conditions improve the selectivity of specific peptides. Besides selectivity of process, the use of PEF and RP as electric field conditions have previously reported to improve process performance by controlling concentration polarization and mineral fouling on IEM mainly while working with food processing [74][60][75].

EUR6 model is the first pilot-scale EDUF configuration used for the fractionation of enzymatically generated peptides to our knowledge. It was the first attempt to analyze the effect of electrodialytic parameters and also electric field conditions on pilot scale EDUF. When compared to results in the literature obtained by using lab-scale EDUF model, the migration rates were lower in the present study. The possible reasons for lower migration rate of peptides and inefficient separation of anionic and cationic peptides can be discussed by explaining the electric field strength, pressure and properties of UFM used in the present study. It is true that electric flow is proportional to electric field strength. An electric field strength of 0.7 V/cm applied in the current study is considerably low for a pilot-scale model and in comparison with previous studies. On the other hand, an applied pressure of 10 psi was found to be more dominant than electric field. As a consequence the migration of peptides was more governed based on the size of peptides than the charge of peptides. This led to decrease in membrane selectivity and it is also further verified by HPLC chromatograms of peptides obtained in recovery compartments which had similar peaks. In addition, the MWCO of ultrafiltration membranes used in the current study were of 100 kDa and it is supposed to be high since the peptides generated from $\beta - Lg$ source on enzymatic hydrolysis were not larger than 1500 Da.

Besides the MWCO of UFM, it is also recommended to account the conductivity of the membrane since it affects the migration rate of peptides. It will also be interesting to study the possible arrangement of EDUF configuration, like number of spacers used and its thickness. Furthermore, it is believed that peptides separation can be efficiently achieved using pilot-scale EDUF in future by optimally controlling: (a) process parameters like electric field strength, controlled pressure (no transmembrane pressure), feed concentration, working pH and solution conductivity, and (b) membrane properties like MWCO, conductivity and

thickness of ultrafiltration membrane. It is expected that industrial reality with EDUF process is very promising for the separation of bioactive compounds when process optimization can be done.

6. References

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